

PATENT
Attorney Docket 2183-5238US

NOTICE OF EXPRESS MAILING

Express Mail Mailing Label Number: EL 740516437US

Date of Deposit with USPS: January 14, 2002

Person making Deposit: Orlena Howell

APPLICATION FOR LETTERS PATENT

for

MOSAIC INFECTIOUS BURSAL DISEASE VIRUS VACCINES

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Title: MOSAIC INFECTIOUS BURSAL DISEASE VIRUS VACCINES

CROSS-REFERENCE TO RELATED APPLICATION

This application is a continuation of International Application Number PCT/NL00/00493 filed on July 13, 2000 designating the United States of America, International Publication No. WO 01/04319 (January 18, 2001), the contents of the entirety of which is incorporated by this reference.

TECHNICAL FIELD

The invention relates generally to biotechnology, and more particularly to Infectious Bursal Disease Virus ("IBDV") vaccines.

BACKGROUND

Infectious bursal disease ("IBD"), an infectious disease among young chickens, was first recognized in 1957 in Gumboro, Delaware, USA and formally documented by Cosgrove (Cosgrove, 1962; Lasher and Shane, 1994). As a result, the disease is often referred to as "Gumboro". Not long after IBD was first reported, it was recognized in poultry populations throughout the world (Lasher and Shane, 1994). IBD is caused by a virus ("IBDV") classified as a *Birnavirus* (Dobos et al., 1979).

Two different IBDV serotypes exist: serotypes I and II (Jackwood et al., 1982; McFerran et al., 1980). Isolates belonging to serotype I are highly pathogenic for chickens. Serotype II isolates, which are mainly recovered from turkeys, have not been reported to induce clinical signs in chickens and are regarded as apathogenic (Ismail et al., 1988). Infectious bursal disease or Gumboro is a highly contagious disease for young chickens, and is responsible for severe losses in the poultry industry. In birds surviving an acute infection, lymphoid cells in the bursa of Fabricius are destroyed, resulting in B-cell dependent immunodeficiency. This causes increased susceptibility to disease caused by otherwise harmless agents. A central role in the pathogenesis of Gumboro is played by the bursa, which is representing the target organ of the virus.

IBDV infections were initially recognized by whitish or watery diarrhea, anorexia, depression, trembling, weakness, and death. This clinical IBD was generally seen in birds between three and eight weeks of age. The course of the disease runs approximately 10 day in a flock. Mortality usually ranges from 0-30 percent. Field reports

suggest that leghorns are more susceptible to IBDV than broiler type chickens. Subclinical IBD was later recognized and is generally considered a greater problem in commercial poultry than the clinical disease. It is generally seen in birds less than three weeks of age. This early infection results in a B-lymphocyte depletion of the bursa of Fabricius. The bird is immunologically crippled and unable to respond fully to vaccinations or field infections. In susceptible chickens, damage caused by IBDV can be seen within two to three days after exposure to virulent virus. Initially, the bursa swells (3 days post-exposure) with edema and hemorrhages and then begins to show atrophy (7-10 days). IBD virus is especially cytopathic to certain B-lymphocytes. The highest concentration of these specific B-lymphocytes is found in the bursa. Destruction of the B-lymphocytes by IBD field virus may result in an incomplete seeding of these cells in secondary lymphoid tissue. As a result of the depletion of B-lymphocytes, surviving birds are immunocompromised during the remaining of their live time.

IBDV is found worldwide, and IBDV specific antibodies have even been found in Antarctic penguins (Gardner et al., 1997). The prevalence of clinical IBD is relatively low compared to the prevalence of subclinical IBD. IBDV is very resistant to common disinfectants and has been found in lesser mealworms, mites, and mosquitoes. These facts correlate with field experience of reoccurring IBD problems on a farm, despite clean-up efforts. Infection with IBDV results in a strong antibody response against IBD, which is capable of neutralizing this virus. Most likely as a result of vaccination, antigenic variant isolates of serotype I were isolated in the Delaware area (USA). These isolates have been shown to cause bursa atrophy in as little as three days post-infection without inflammation of the bursa. Despite their change in antigenicity these antigenic variants do not form a distinct serotype. After the occurrence of antigenic variant IBDV isolates in the USA, the poultry

- industry in European countries was hit by outbreaks of IBD caused by a very virulent serotype I IBDV (vvIBDV) (Berg et al., 1991; Chettle et al., 1989; Kouwenhoven and Van den Bos, 1995). These very virulent field isolates were capable of establishing themselves in the face of high levels of maternal antibodies which normally were protective. These vvIBDV cause more severe clinical signs during an outbreak and are now found globally (e.g. Europe, Japan, Israel and Asia).
- IBDV belongs to the family of Birna viruses which include Infectious Bursal Disease Virus (IBDV) isolated from chickens, Infectious Pancreatic Necrosis Virus (IPNV) isolated from Fish, Drosophila X Virus (DXV) isolated from fruit fly, and Tellina virus (TV) and Oyster Virus (OV) both isolated from bivalve molluscs (Dobos et al., 1979). Birna viruses have a dsRNA genome which is divided over two genome segments (the A- and B-segment). The A-segment (3.3 kbp) contains two partly overlapping open reading frames (ORFs). The first, smallest ORF encodes the non-structural Viral Protein 5 (VP5, 17 kDa). The second ORF encodes a polyprotein (1012 amino acid, 110 kDa), which is autocatalytically cleaved. The exact position of these cleavage sites is unknown. From SDS-Page analysis of *in vitro* translated IBDV RNA it is known that the polyprotein is rapidly cleaved into three proteins: pVP2 (48 kDa), VP4 (29 kDa) and VP3 (33 kDa). During *in vivo* virus maturation pVP2 is processed into VP2 (38 kDa), probably resulting from site-specific cleavage of the pVP2 by a host cell encoded protease (Kibenge et al., 1997). VP2 and VP3 are the two proteins that constitute the single shell of the virion. The B-segment (2.9 kbp) contains one large ORF, encoding the 91 kDa VP1 protein. This protein contains a consensus RNA dependent RNA polymerase motive (Bruenn, 1991).
- Furthermore, this protein has been reported to be linked to the 5'-ends of the genomic RNA segments (Viral Protein genome-linked, VPg). The nucleotide sequence of internal parts of a large number of IBDV isolates of classical,

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antigenic variant or very virulent origin has been determined, and deposited in several databases such as GenBank. Furthermore Mundt and Muller (Mundt and Muller, 1995) have determined the 5'- and 3'-termini of several IBDV isolates (CU-1, CU-1M, P-2 and 23/82), and by combining the internal and terminal sequences, Mundt and Muller established the complete nucleotide sequence of a serotype I A-segment (3261 bp) and B-segment (2827 bp). This provided the way to generate an infectious (recombinant) copy (rIBDV) of IBDV serotype I, by knowing the complete sequence dsRNA sequence of IBDV genome and by using one of several methods to generate infectious copy virus (see for example Boyer et al, Virology 198:415-426, 1994), Mundt and Vakharia indeed produced infectious rIBDV serotype I from cDNA (Mundt and Vakharia, 1996). Full length cDNA of a serotype I IBDV, preceded by a T7 promoter, was thereby used as a template for T7 RNA polymerase using a method described by Weiland and Dreher (Weiland and Dreher, 1989). The *in vitro* generated mRNA, containing a cap-structure at its 5'-end, was subsequently transfected into eukaryotic cells (VERO cells) using a liposome formulation (Lipofectin, GibcoBRL). The supernatant of the transfected cells contained infectious rIBDV after incubation during 36h in the CO₂ incubator at 37° C (Mundt and Vakharia, 1996; (WO 98/09646)). In addition, Lim et al. introduced two amino acid mutations (D279N and A284T) into the cDNA of vvIBDV isolate HK46 (Lim et al., 1999). These mutations were most probably based on data of Yamaguchi et al. (Yamaguchi et al., 1996), which showed that these specific mutations were found in two independent experiments in which very virulent IBDV isolates lost their very virulent character by adaptation and growth on primary CEF cells. Lim et al obtained a rIBDV isolate which possessed the phenotype of a CEF-culture adapted isolate, i.e. a rIBDV isolate which can be propagated, i.e. is able to infect, multiply and be released for further replication, in vvIBDV non-

permissive cells such as CEF cells. Note worthy, Lim et al. were unable to produce an infectious vvIBDV isolate using the unmodified cDNA of the HK46 isolate (Lim et al., 1999). Furthermore, although cDNA of IBDV can be used to produce infectious IBDV, the exact mechanism of replication has not been elucidated yet. Data exist which are in support of a semi-conservative genome replication model for Birnaviridae (Bernard, 1980; Mertens et al., 1982).

Now and then IBDV variants are detected in the field or are created in cell-culture in the laboratory (Muller, 1987) that are genetic re-assortments of serotype I and II strains of IBDV, in that they contain one genomic segment derived from the one serotype, and another segment derived from the other serotype. Such segment reassorted (srIBDV) strains (also called chimeric IBDV) not only occur in nature, but have recently been generated from cDNA as a well, by Vahkaria and Mundt (WO 98/09646). Vaccination using attenuated field isolates worked sufficiently well until antigenic derivatives were found in the Delaware region of the USA starting in 1985 (isolates Del A, D, G and E) (Snyder, 1990). These field isolates were missing an important virus neutralizing epitope. The change of this epitope is characterized by the lack of binding of the virus neutralizing monoclonal antibody (Mab) B69 (Snyder et al., 1988a). The antibodies induced by vaccination with classical IBDV vaccines appeared to be less protective against these antigenic IBDV variants. Inactivated vaccines based upon antigenic IBDV variants were subsequently produced and were found to protect effectively against these antigenic variants of IBDV. After the Delaware variant a second antigenic variant IBDV was isolated. This variant was recovered from the Delmarva region (USA) and was referred to as the GLS variant. The GLS variant is characterized by the absence of epitopes for both the virus neutralizing Mab B69 and R63 (Snyder et al., 1988b). After identifying these antigenic variants, a large survey was performed within the USA by using a panel of nine Mabs against

IBDV. This survey yielded an additional antigenic variant: the DS326 variant. This antigenic variant is characterized by the absence of epitopes for Mab 179 and BK44, in addition to those for Mabs B69 and R63 (Snyder, 1990). No further reports of antigenic variants have been published in the USA or in other parts of the world. Whether this is due to non-existence of new variant IBDV isolates or whether new antigenic variants just have not been detected due to the lack of extensive surveys or the lack of discriminating monoclonal antibodies is unclear.

The nucleotide sequence of the polyprotein encoding part of the A-segment of the Del, the GLS and the DS326 antigenic variant IBDV isolates has been determined (Vakharia et al., 1994). Most of the amino acid changes were found in a specific region of the VP2 protein, the so-called hypervariable region. Furthermore it was found that the epitopes which are capable of inducing neutralizing antibodies are conformation dependent and are clustered in the hypervariable region. This region consists of a domain with a high hydrophobicity index (amino acid 224 to 314 of pVP2, corresponding with amino acid 224 to 314 of the polyprotein) which is flanked by two small hydrophilic regions, each spanning about 14 amino acids (Vakharia et al., 1994; Heine et al., 1991). Amino acid substitution both within the hydrophobic region and within the hydrophilic regions might be involved in the antigenic variant character of these isolates.

After the problems caused by the antigenic variant IBDV isolates in the USA, the poultry industry in Europe was affected by very virulent IBDV (vvIBDV) isolates (Berg et al., 1991; Chettle et al., 1989). The vvIBDV isolates cause more severe clinical signs during an outbreak and are able to break through levels of antibodies which are protective against classical IBDV isolates. The molecular determinants which distinguish vvIBDV from classical IBDV isolates are not exactly known. It is known however, that the pathogenicity of cell culture adapted very virulent IBDV isolates is

severely reduced, compared with the non-adapted parental isolates (Yamaguchi et al., 1996). The correlation between CEF-adaptation and loss of the very virulent phenotype is likely to be due to the change in target cell tropism of the adapted virus. This change in cell tropism may be due to the loss of bursa cell receptor binding capability of the cell culture adapted very virulent IBDV isolate. Another possibility is that the cell culture adapted very virulent IBDV isolate is able to infect non-bursa cells, resulting in large reduction of IBDV load in the primary target cells (bursa cells). From the published results (Yamaguchi et al., 1996), it is clear that a recombinant IBDV (rIBDV) which is based upon the cDNA of a cell culture adapted very virulent isolate will never yield a vaccine which meets the demands of being able to break through high levels of maternal antibodies and induce a high enough immune response.

No specific antibodies, that exclusively recognize the vvIBDV isolates have been described yet (Eterradossi et al., 1997)). The lack of discriminating antibodies makes direct diagnosis difficult. Most attention has been given to sequence comparison between the hypervariable region of VP2 of classical isolates and of very virulent isolates. Sequence analysis of the vvIBDV isolate UK661 showed that only three unique (i.e. not found in non-vvIBDV isolates) amino acid substitution are present within the hypervariable region of the VP2 protein. One amino acid substitution is present within the remaining part of the pVP2 protein, while 5 unique amino acid mutations are present within the VP4 encoding part of the polyprotein and 6 in the VP3 encoding part. (Brown and Skinner, 1996). The smaller ORF of the UK661 isolate A-segment, encoding the VP5 protein, contains 2 unique amino acid substitutions. Additionally 16 unique amino acid substitutions were found in the VP1 protein encoded by the B-segment of this vvIBDV isolate. The virulent phenotype of the vvIBDV might be influenced by each of the found amino acid substitutions, and even (silent)

nucleotide substitutions within the coding or non-coding parts of either the A- or B-segment may contribute to the altered phenotype of the vvIBDV isolates in comparison with the classical or antigenic variant isolates. Serial

5 passage on embryonated eggs of a vvIBDV isolate (OKYM) resulted in the appearance of a derivative isolate (OKYMT) which is able to grow on Chicken Embryo Fibroblast (CEF) cells and has lost its virulence. This adaptation was reported to be the result of 7 nucleotide

10 substitutions in the polyprotein encoding part of the genome. Whether additional nucleotide substitutions (or deletions) were present in remaining parts of the A- or B-segment (e.g. untranslated regions, VP1 encoding region, and VP5 encoding region) was not determined

15 (Yamaguchi et al., 1996). The reported nucleotide substitutions result in 5 amino acid substitutions. Three of these amino acid substitutions were located in the hydrophobic part of the hypervariable region (I256T, D279N, A284T) of VP2, one in the hydrophilic part located

20 downstream of the hypervariable region (S315F) of VP2, and one in VP3 (A805T) (Yamaguchi et al., 1996). In an independent experiment, Yamaguchi et al. found that the adaptation of vvIBDV isolate TKSM into TKSMT resulted also in the A284T and D279N substitutions. The A284T

25 substitution correlated in their analysis completely with adaptation onto CEF cells and loss of virulence. The D279N substitution was also present in both CEF-adapted vvIBDV isolates (OKYMT and TKSMT) and is potentially also important for growth on CEF cells and loss of

30 virulence. The non-CEF adapted, classical IBDV isolate GBF-1 has on the other hand an asparagine at position 279, in combination with alanine at position 284 and cannot grow on CEF cells, so the single substitution D279N does not account loss of virulence and growth on

35 CEF cells. The amino acid changes in the VP2 apparently allow the modified IBDV to propagate on cells which do

not have a receptor for wild type IBDV. Cells possessing a wild-type IBDV receptor such as bursa cells are susceptible for classical and vvIBDV isolates. Recently is was shown that amino acid substitution, A284T in combination with D279N is indeed enough to turn a non-CEF-adapted very virulent IBDV isolate into a CEF-adapted isolate. Lim et al. introduced these two amino acid substitutions into the A-segment cDNA of vvIBDV isolate HK46 (Lim et al., 1999). After transfection of this cDNA, Lim et al obtained a rIBDV isolate which possessed the phenotype of a CEF-culture adapted isolate, i.e. a rIBDV isolate which is able to infect and multiply in CEF cells. The virulence of this rIBDV isolate was not assessed in chickens. Note worthy, Lim et al. were unable to produce a recombinant infectious vvIBDV isolate using the unmodified cDNA of the HK46 isolate (Lim et al., 1999).

The goal of vaccination against IBD is prevention of subclinical and clinical IBD and the economic aspects of each. Effective vaccination for IBD can be divided into the following categories:

- Protection of the developing bursa in broilers, breeders and layers.
- Prevention of clinical disease in broilers, breeders and layers.
- Priming and boosting of breeders.

To minimize the immunosuppressive effects of IBDV, the young chick must be protected. Protection of the very young can be achieved through high enough levels of maternal antibodies passed from the breeder hen to her progeny. Vaccination of the very young chick itself may not be successful since onset of protection after vaccination is between three and five days. When a bird, lacking maternal antibodies against IBDV, is exposed to a pathogenic IBDV field strain, damage will occur within 24-48 hours.

Generally the early vaccinations of the breeders serve as priming. In most situations, this single vaccination is not considered to be adequate. Boosting is the term commonly associated with the administration of a final IBDV vaccination prior to the onset of lay. This is done to increase the circulating antibody in the hen and hence the maternal antibodies in the progeny. Both inactivated (oil emulsion vaccine) and live vaccines (IBDV) have been used for this purpose. The use of a live vaccine in an older bird will result in an increase of antibodies; however, large variations in antibody titers are often seen. These variations result in progeny becoming susceptible to field challenge from as early as a few days after hatching to 21 days after hatching. The use of inactivated IBDV vaccines gives a higher antibody titer as well as a decrease of variation between antibody titers of birds belonging to the same flock. The levels of maternal antibodies necessary to neutralize IBD vary with the invasiveness and pathogenicity of the field strain. In practical terms, if a very virulent IBDV isolate is present, higher maternal antibody levels are desired (see Table 1 for an overview of virulence of field isolates and strength of vaccines). Yet, for effective vaccination, avoiding interference with maternal antibodies is needed to induce a good immune response. Clinical IBD is typically seen between three and six weeks of age. The immune response of the chick must be stimulated as the passive protection is declining. The timing of the active vaccination may be estimated by the breeder or chick titer and the half-life of antibodies of approximately 3.5 days (De Wit and Van Loon, 1998; Kouwenhoven and Van den Bos, 1995). The levels of maternal antibodies tend to vary within a population. This variation might be a result of variation in the antibodies levels of the breeder hen. Also the mixing of progeny from several breeder flocks (e.g. combination of breeders of different age; breeders vaccinated with live vaccine and those with oil emulsion

vaccine) results in variation of IBDV antibodies between chick belonging to the same flock. If the coefficient of variation (CV) in mean maternal antibody titers is too wide, it may be recommended to vaccinate twice (with a
5 10-day interval) or to vaccinate early with a hot vaccine (in the presence of a high antigenic pressure).

The average titer of antibody against IBDV in a flock will decline in time (Fig. 1). As a result of the decrease in average antibody titers, an immunity gap
10 will occur. The best results are obtained if the immunity gap is as short as possible and is as early as possible, with a minimum of 2 weeks after hatching. There should be at least sufficient immunity after active vaccination at the age of 4 weeks, since many handlings occur in the
15 houses from that time point with risks of introducing field virus. Therefore farmers like to vaccinate at 2 weeks or even before. Intermediate vaccines are often unable to break through the average IBDV antibody titer of the broiler at two weeks after hatching (Fig. 1). If
20 there is a high variation in mean maternal antibody titers, some chicks will be effectively vaccinated with intermediate vaccines, others not. To circumvent those problems, hot vaccines are being used. A drawback of usage of hot vaccines is that the bursa of chickens with
25 low to moderate maternal antibody titers will be (partly) damaged.

There is a wide variety of IBDV vaccines available. Important aspects in vaccination strategies are the ability of the virus to replicate in the face of maternal
30 antibody (invasiveness of the vaccine) and the spectrum of antigenic content (including antigenic variants). The ability of a vaccine virus to replicate in the face of maternal antibodies allows live vaccines to be categorized into three main groups: mild, intermediate,
35 and intermediate plus or hot vaccines (see Table 1). The initial vaccines for IBD were derived from classical IBDV isolates. These vaccines were moderately pathogenic IBDV strains with low passage numbers in embryonated eggs. These were often used in breeder programs to induce

high levels of circulating antibodies. However, when given to a young bird with moderate or low levels of maternal antibodies, these vaccines could cause extensive bursal atrophy resulting in immunosuppression. Mild
5 vaccines were subsequently developed to be used in these young birds. The attenuation of classical IBDV was done in tissue culture systems. Traditionally, attenuated strains for vaccines are generated by adapting IBDV strains to chicken embryoblast (CEF) cells or other
10 appropriate cells or cell lines through serial passages. These vaccines are not immunosuppressive even when used in birds having no maternal antibodies. However, moderate and high levels of antibodies easily neutralize them. As breeder programs developed (including the use of
15 adjuvant, inactivated vaccines), higher levels of maternal antibodies were generated in progeny. This reduces the effectiveness of these mild vaccines.

Intermediate strength vaccines were to overcome the inadequacies of the mild vaccines. Some of the
20 intermediate vaccines were developed by cloning a field isolate on chicken cell cultures. Intermediate strength vaccines are capable of establishing immunity in birds with moderate levels of maternal antibodies. These vaccines will cause some bursal atrophy in birds without
25 maternal antibodies, but are considered not immunosuppressive.

Hot (strong) or intermediate plus vaccines were developed after the first outbreaks with vvIBDV. These vvIBDV isolates could break through higher levels of
30 maternal immunity than the vaccines that were on the market at that time. Vaccination with intermediate vaccines came always too late in situations with high infection pressure with vvIBDV. Hot vaccines consist of vvIBDV strains with low to moderate passage in
35 embryonated eggs or bursa derived IBDV of chickens infected with vvIBDV isolates. Adapting vvIBDV on cells traditionally used for the generation of vaccines in general fails, since either these cells are non-permissive for vvIBDV, or, when adapted to said cells,

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the vvIBDV in question had lost its very virulent character, making it useless for hot or intermediate plus vaccine. Hot or intermediate plus vaccines are desirably able to circumvent maternal immunity at an earlier age than intermediate vaccines but spread more within a flock. If intermediate plus and hot vaccines are used in chickens with moderate to high levels of maternal antibodies, there is no negative side effect on the bursa (Kouwenhoven and Van den Bos, 1995). If these vaccines are used in chickens with low to moderate levels of maternal immunity, this causes depletion of lymphoid cells in the bursa and a severe depletion of peripheral blood-B cells is found (Ducatelle et al., 1995). Although a recovery of bursal function has been observed, these vaccines should be used with precautions.

Live vaccines must be given in a way in which the virus will preferably reach the bursa where it will quickly multiply and induce an immune response. Possible routes for application of live vaccines include drinking water, spray, subcutaneous and in ovo. Inactivated IBD vaccines are used in broiler breeders. They differ in some of the same ways as live vaccines. Their efficacy depends upon the spectrum of antigens they contain. Injectable oil-emulsion products may be given subcutaneously or intramuscularly.

A continuous monitoring of the field situation using an integrated quality control scheme including serology, can be a valuable tool for continuously adapting preventive vaccination programs to changing epidemiological conditions. Also a continuous follow-up of the epidemiological situation will allow to anticipate the development of major epidemics (Ducatelle et al., 1995). However, the ability of diagnostic laboratories to monitor IBD with meaningful definitive data is difficult. Serology is important but can be confusing when all birds monitored from commercial broiler flocks have high levels of the same spectrum of circulating antibodies. Field evaluations of broilers to monitor the status of IBD are

highly subjective: it is difficult to discriminate antibody titers obtained after vaccination from those induced by IBDV field infections. If it were possible to discriminate between IBDV antibody response to field virus and IBDV vaccination it is possible to have 'early warning' systems and to start IBDV eradication programs if desired. Only when there is a known difference between the antibody response to the used IBDV vaccine and IBDV field isolates, defined conclusion about whether (sub)clinical signs of IBDV are the result of live IBDV vaccination or of IBDV field isolates can be made.

The invention provides infectious recombinant Infectious Bursal Disease Virus (rIBDV) essentially incapable of growing in a cell that is not derived from a bursa cell or another cell comprising a wild-type IBDV receptor (a non-bursa-cell). A bursa is lymphoid organ, mostly comprising cells that are related to the immune system. In particular, it comprises lymphocytes or lymphocyte precursor cells of sometimes the T-cell- but mainly the B-cell-type, and cells derived thereof, in close relation with monocytes or monocyte derived cells such as macrophages, and also with follicular dendritic cells and antigen presenting cells. In particular, the invention provides rIBDV that is essentially incapable of growing in a cell not listed among above bursa cells or cells derived thereof, such as dendritic cells, monocytes, lymphocytes or cells derived thereof. Herewith the invention provides an rIBDV having retained an important characteristic, in that, an comparison with commonly attenuated IBDV strains, it can not or only little grow in non-bursa cells, such as the well known CEF, QM5 or VERO cells, or other cells that are commonly used for propagating attenuated strains of IBDV. In particular, the invention provides an rIBDV essentially incapable of growing in a non-B-cell derived cell. Essentially incapable of growing herein means that the isolate in question is not or only little capable to

infect, multiply or be released for further replication. No such rIBDV isolates exist prior to this invention, all previous rIBDV isolates known grow in non-bursa-cell derived cells such as CEF cells (WO98/09646; Lim et al., 1999), thereby for example having lost those very virulent characteristics essential for maintaining in a vaccine strain designed to face above identified problems.

In a preferred embodiment the invention provides in infectious rIBDV having retained at least part of the very virulent characteristics of a very virulent Infectious Bursal Disease Virus (vvIBDV) needed to provide protection against vvIBDV. In particular, vvIBDV is provided that is essentially incapable of growing in a non-bursa-cell derived cell. In particular, as for example demonstrated in the detailed description, the invention provides an rIBDV essentially incapable of growing in a CEF cell, a VERO cell or a QM5 cell, except of course in those CEF, VERO, QM5, or related cells having been provided with the necessary means (such as transgenic receptor or replication system derived from a bursa-cell or other characteristic of B-lymphoid cells which support the propagation of IBDV in a essentially non-lymphoid cell if this characteristic is present in such a non-B-lymphoid cell) needed for replication of classical or very virulent IBDV.

Furthermore, the invention provides an rIBDV wherein the amino acid sequence of protein VP2 comprises no asparagine at amino acid position 279, but for example comprises an amino acid particular for a strain with a very virulent character, such as with aspartic acid at amino acid position 279. Such rIBDV strains as provided by the invention have retained at least part of the very virulent characteristics of vvIBDV, as well as an rIBDV according to the invention wherein the amino acid sequence of protein VP2 comprises no threonine at amino acid position 284, but for example comprises an amino acid particular for a strain with a very virulent

character, such as with alanine at amino acid position 284.

In a preferred embodiment, the invention provides an rIBDV according to the invention wherein the amino acid sequence of protein VP2 at least comprises a stretch of amino acids from about position 279 to 289, preferably from about position 229 to 314, most preferably from about position 214 to 328 as found in a vvIBDV isolate such as shown in Table 8.

10 The invention furthermore provides a method for obtaining an infectious recombinant copy Infectious Bursal Disease Virus (rIBDV) essentially incapable of growing in a non-bursa-cell derived cell or having at least part of the very virulent characteristics of a very
15 virulent Infectious Bursal Disease Virus (vvIBDV) comprising transfecting at least one first cell with a nucleic acid such as a cDNA or RNA comprising an IBDV genome at least partly derived from a vvIBDV, incubating said first cell in a culture medium, recovering rIBDV
20 from said transfected first cell or said culture medium, propagating said recovered rIBDV in at least one second cell which is permissive for said vvIBDV. A vaccine derived of the recombinant virus as described is also part of this invention. Also a vaccine comprising a
25 chemically or physically inactivated recombinant virus or parts thereof is part of this invention.

Also the attenuated derivatives of initially produced recombinant very virulent IBDV are part of this invention. Such a virus can be attenuated by known
30 methods including serial passage, removing specific nucleic acid sequences, or by site directed mutagenesis. Physiologically acceptable carriers for vaccines of poultry are known in the art and need not be further described herein. Other additives, such as adjuvants and
35 stabilizers, among others, may also be contained in the vaccine in amounts known in the art. Preferably, adjuvants such as aluminum hydroxide, aluminum phosphate,

plant and animal oils, and the like, are administered with the vaccine in amounts sufficient to enhance the immune response to the IBDV. The vaccine of the present invention may also contain various stabilizers. Any
5 suitable stabilizer can be used including carbohydrates such as sorbitol, mannitol, starch, sucrose, dextrin, or glucose; proteins such as albumin or casein; and buffers such as alkaline metal phosphate and the like. A stabilizer is particularly advantageous when a dry
10 vaccine preparation is prepared by lyophilization. The vaccine can be administered by any suitable known method of inoculating poultry including nasally, ophthalmically, by injection, in drinking water, in the feed, by exposure, and the like. Preferably, the vaccine is
15 administered by mass administration techniques such as *in ovo* vaccination, by placing the vaccine in drinking water or by spraying the animals' environment. When administered by injection, the vaccines are preferably administered parenterally. The vaccine of the present
20 invention is administered to poultry to prevent IBD anytime before or after hatching. Poultry is defined to include but not be limited to chickens, roosters, hens, broilers, roasters, breeders, layers, turkeys and ducks. Examples of pharmaceutically acceptable carriers are
25 diluents and inert pharmaceutical carriers known in the art. Preferably, the carrier or diluent is one compatible with the administration of the vaccine by mass administration techniques. However, the carrier or diluent may also be compatible with other administration
30 methods such as injection, eye drops, nose drops, and the like.

As explained above, there is need for an IBDV vaccine that can protect against field infections with IBDV, and preferably against very virulent IBDV (vvIBDV).
35 It is clear that vaccines derived from attenuated classical strains and not from very virulent strains will

not be able to sufficiently protect. However, as explained above, simply by adapting and cultivating a vvIBDV strain on a cell or cell-line, such as VERO, CEF or QM5, as one skilled in the art would first do in order to obtain a vaccine strain from a vvIBDV strain, reduces its virulent phenotype such that no sufficient protection is to be expected. Therefore, a vaccine strain is needed that has at least partly maintained the very virulent or hot character, in order to provide sufficient protection, however, paradoxically, such a desirable vaccine strain would most likely not be able to be sufficiently or substantially be propagated on appropriate cells, such as non-bursa-cell derived VERO, CEF or QM5, deemed needed to obtain said vaccine. In a preferred embodiment, the invention provides a method wherein said first cell is a non-bursa-cell derived cell non-permissive for said vvIBDV, preferably wherein said first cell has additionally been provided with a helpervirus or a viral protein (herein T7-polymerase is used) derived thereof. With the help of such a cell comprising a properly selected helpervirus, e.g. expressing distinct IBDV or Birna virus viral proteins, or of a cell expressing said IBDV or Birna virus viral proteins, (also called a complementary cell) also now defective or deficient rIBDV can be made.

The invention therewith also provides a method to generate infectious Infectious Bursal Disease Virus, by combining cDNA sequences derived from very virulent IBDV (vvIBDV) isolates with cDNA sequences derived from either serotype I classical attenuated IBDV isolates, serotype I antigenic variants of IBDV, or serotype II IBDV isolates, wherein said infectious copy recombinant Infectious Bursal Disease Virus having retained at least part of the very virulent characteristics of a very virulent Infectious Bursal Disease Virus has at least retained the incapacity to substantially be propagated on a vvIBDV non-permissive cell such as a QM5 or CEF cell.

Preferably, a method as provided by the invention provides a vaccine comprising an IBDV genome wherein

parts of segments A and/or B derived from a vvIBDV are used combined with parts of segments A and/or B derived from an attenuated IBDV, such as attenuated serotype I or I IBDV. Such a rIBDV is herein also called a mosaic IBDV (mIBDV). Herein we show that (mosaic) vvIBDV can be generated from cDNA by transfection of non-susceptible cells followed by amplification of the cDNA-derived rIBDV on susceptible cells. The method provided herein provides a method to generate vvIBDV from the cloned, full length cDNA of a vvIBDV isolate (see Table 5 and 6). After transfection of QM5 cells with cDNA of vvIBDV it is essential that propagation of the generated vvIBDV virus takes place on cells which are permissive for vvIBDV. These permissive cells can for example be found among Bursa-cell derived cells such as primary bursa cells, in chicken in embryo cells, chicken embryo's, or young chickens. Using the method described herein we have for example produced recombinant D6948 (rD6948) using the cDNA derived from the very virulent D6948 IBDV isolate. This rD6948 isolate has the same virulence as the parental D6948 isolate (Table 6).

Preferably, the invention provides a method wherein a permissive second cell is a primary bursa cell, thereby allowing initial propagation of the desired vaccine virus. As explained above, there is a need for a vaccine capable of breaking through maternal immunity of young chickens at an early stage. A desired vaccine should preferably be able to induce a high level of protection in vaccinated young chickens, and should therefore be as immunogenic as very virulent viruses or be almost as immunogenic.

The invention furthermore provides a method to engineer recombinant mosaic IBDV (mIBDV) vaccine which has one or more of the desired phenotypes, i.e. i) being able to break through high levels of maternal antibodies in young chickens and being highly immunogenic, ii) having a reduced pathogenicity compared to wild type very virulent IBDV isolates. In one embodiment, the invention provides an infectious mosaic IBDV (mIBDV) comprising a

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rIBDV wherein at least one genome segment comprises nucleic acid derived from at least two different Birna virus isolates, when preferred wherein at least one of said isolates is a vvIBDV characterised by its incapacity to substantially be propagated on a vvIBDV non-permissive cell such as a VERO, QM5 or CEF cell and/or characterised by its capacity to substantially be propagated on a vvIBDV permissive cell such as a primary bursa cell. For example, the invention provides mIBDV which consists partly of the genome derived from a classical attenuated isolate (such as CEF94) and partly derived from the genome of a vvIBDV isolate (such as D6948). A recombinant mosaic IBDV (mIBDV), made on the basis of infectious cDNA derived from a very virulent IBDV isolate (D6948), and combined with defined parts of cDNA derived from a cell culture adapted, serotype I, classical IBDV isolate (CEF94) results in a mIBDV isolate which has a reduced pathogenicity compared to wild-type vvIBDV isolates which are essentially not replicating on non-B-lymphoid derived cells.

Furthermore specific nucleotide substitutions which either do or do not lead to amino acid mutations, or deletion of specific parts of the IBDV genome again leads to an altered phenotype of the generated mIBDV. For example, the replacement of the pVP2 coding region of CEF-94 cDNA with the corresponding region of cDNA of D6948 yielded plasmid pHB36-vvVP2. This plasmid was subsequently transfected into FPT7 (Britton et al., 1996) infected QM5 cells in combination with pHB-34Z. Supernatant of these transfected QM5 cells was subsequently transferred to fresh QM5 cells. None of these QM5 cells reacted positively in an IPMA using specific antibodies for the VP3 protein of IBDV. On the other hand, primary bursa cells, after being overlaid with supernatant of the transfected cells, reacted positively in the same IPMA. The functional feature of being able to enter permissive cells such as QM5 cells is apparently located in the pVP2 coding region of the A-segment. This invention provides a method to generate

recombinant vvIBDV (such as rD6948) having a pVP2 sequence exactly as found in a wild-type vvIBDV (here D6948). All very virulent isolates of which the pVP2 sequences has been described thus far have an alanine at position 284 and cannot or only little be propagated on CEF cells (see Table 7 and 8). On the other hand, when a threonine is present at position 284, propagation on CEF cells is possible, but this is associated with the lack of a very virulent phenotype (see Table 7 and 8). Herein we describe a method to generate infectious recombinant IBDV (rIBDV) having the nucleotide sequence of a wild-type very virulent IBDV isolate, including the alanine codon for amino acid 284, and being unable to be propagated on CEF cells. Furthermore, in our rD6948 isolate we have an aspartic acid present at position 279 in stead of a asparagine commonly found for avirulent IBDV isolates which can be propagated on CEF cells (Table 7 and 8). The rD6948 is truly a very virulent rIBDV, as it is unable to grow on CEF cells (Table 5), and induces similar clinical signs and mortality as wild-type very virulent D6948 isolate (Table 6). Although mIBDV isolate (mCEF94-vvVP2) did, in contrast to the D6948, rD6948 and srIBDV-DACB isolates (also having a functional VP2 protein derived from vvIBDV, see Table 6), not cause any mortality in a 9-days course or body weight loss, it caused the same reduction in bursa weight after 9 days post-infection as the wild-type very virulent D6948 isolate.

In yet another embodiment, the invention provided a mosaic IBDV according to the invention wherein at least one of said isolates is a serotype II IBDV. Such a mIBDV, preferably lacking at least one immunodominant epitope specific for a serotype I IBDV as well is a (r)D6948 derived vaccine virus such as mD6948-s2VP3C1, also having a functional VP2 protein derived from vvIBDV, allowing vaccination with a marker vaccine. Vaccination with a IBDV marker vaccine and subsequent testing with a

corresponding diagnostic test enables the discrimination between antibodies induced by the vaccine and by infection with IBDV field isolates. This mIBDV can be differentiated from all other known wild-type IBDV isolates, either belonging to serotype I or serotype II, for example by using a specific set of monoclonal antibodies. The generation of mIBDV from serotype I and II cDNA provides such a mIBDV marker vaccine that induces a serological response in chickens that can be differentiated from the serological response induced by IBDV field strains. The marker vaccine provided by the invention, lacking at least one immunodominant epitope, preferably a serotype I epitope, enables the discrimination between vaccinated and infected animals by means of a diagnostic serologic test. Such a mIBDV marker vaccine is preferably based upon vvIBDV and contains specific sequences originating from classical serotype I or serotype II IBDV. Such a mIBDV marker vaccine has one or more of the following characteristics: i) It induces a protective immune response against vvIBDV field viruses despite high levels of maternal antibodies. ii) It has a reduced pathogenicity compared to vaccines based upon wild-type vvIBDV. iii) It for example misses at least one serotype I specific antigen which enables the serological discrimination of the mIBDV marker vaccine from all serotype I IBDV isolates.

Also the invention provides a method to produce or generate tailor made vaccines against specific antigenic variants of IBDV by incorporating the specific amino acid changes in a mIBDV vaccine virus. Depending on the composition, these mosaic IBD viruses (mIBDV) possess different phenotypes and different antigenic properties. A specific mutation in one of the viral proteins can have a profound effect on IBDV viability. We found that this is true in case of a single nucleotide substitution, leading to a single amino acid mutation in VP4 (V582A). No rIBDV could be rescued from cDNA when this particular nucleotide substitution was present. Not only mutations within the VP4 encoding region itself, but also mutations

or deletions in the region of the cleavage sites (pVP2-VP4 and VP4-VP3) may have a negative effect on replication of rIBDV. Mutations in the other viral proteins, or even deletion of an entire viral protein (i.e. VP5) influences the replication and or virulence as well. Two groups have constructed an VP5 minus rIBDV isolate, by introducing mutations in the cDNA of an CEF-adapted D78 IBDV isolate (Mundt et al., 1997; Yao et al., 1998). Apparently the VP5 protein, which is a non-structural protein, is also a non-essential protein. Yao et al. reported that inactivation of the ORF for VP5 (replacement of the startcodon by a stopcodon) yielded infectious rIBDV (rD78NS which grows to slightly lower titers (*in vitro*) than rD78, while Mundt et al. reported that inactivation of the ORF for VP5 (replacement of the startcodon by a arginine codon) yielded a rIBDV (IBDV/VP5-) which is able to grow to the same titers (*in vitro*) as the parental isolate. Furthermore Yao et al. reported that rD78NS has a decreased cytotoxic and apoptotic effects in cell culture (*in vitro*) and has a delay in replication compared to the parental isolate (*in vivo*), and failed to induce any pathological lesions or clinical signs of disease in infected chickens.

Mutations or deletions in the mIBDV cDNA yields a mIBDV with a desired phenotype, i.e. mIBDV which is based on a very virulent isolate but which has a reduced ability to replicate and hence an reduced pathogenicity. The introduction of cDNA sequences from a serotype II, cell culture adapted, IBDV isolate (TY89) into the mosaic virus gives us yet another opportunity to generate marker mIBDV vaccine which can be discriminated from wild-type serotype I IBDV, for example by using specific monoclonal antibodies. Such mIBDV can be used to induce an antibody spectrum, which differs from the spectrum induced by IBDV field isolates. This enables the development of a serologic test to determine whether IBDV antibodies are the result of live mIBDV vaccination or of infection with

IBDV field isolates. For example, the mCEF94-s2VP3C virus is recognized by serotype II specific VP3 antibody (Mab T75) while it is also recognized by a serotype I specific VP2 antibody (Mab 1.4). This particular rIBDV is, on the other hand, not recognized by a serotype I specific VP3 antibody (Mab B10). No apparent difference is present between the replication of mCEF94-s2VP3C and rCEF94, indicating that the exchange of the VP3C-terminal part does not lead to major changes in replication ability in QM5 cells. When, on the other hand, the complete VP3 encoding region was exchanged we observed a severe reduction in replication ability of the resulting virus (mCEF94-s2VP3). On the other hand, mCEF94-s2VP3N was not reacting with Mab C3 (VP3, serotype I) while it is fully reacting with Mab B10 (VP3, serotype I) and only partially with Mab T75 (VP3, serotype II). Replication of this mosaic IBDV on CEF cells is reduced compared to rCEF94. From the generated mIBDV, based on cDNA derived from serotype I (CEF94) and serotype II (TY89), it is clear that a serological marker based on VP3 has been identified. The replacement of the cDNA of (part of) VP3 of serotype I for the corresponding part of serotype II, leads to a unique combination of IBDV antigens within one mIBDV isolate. An mIBDV isolate based on this combination of antigens can be used as an IBDV-marker vaccine.

The introduction of the VP3 C-terminal part of TY89 (Serotype II) into the cDNA of D6948 yielded a mosaic IBDV (mD6948-s2VP3C1) which has a reduced virulence (no mortality, no body weight loss) compared to D6948 or rD6948 (Table 6). This mIBDV, or a comparable isolate which is more or less virulent, is also advantageously used as an IBDV marker vaccine to prevent infections with very virulent IBDV field isolates.

Furthermore, the invention provides using site-specific mutagenesis techniques to introduce any desired nucleotide mutation within the entire genome of mIBDV. Using this technique allows adapting mIBDV vaccines to

future antigenic variations by including any mutation that has been found in antigenic variant IBDV field isolates. Furthermore, it is provided by the invention to exchange part of the genomic sequence of IBDV with the corresponding part of a Birna virus belonging to another genus (e.g. DXV, IPNV, OV, TV). Herewith, the invention provides new mosaic Birna (mBirna) viruses which have new characteristics resulting in new recombinant vaccines for IBDV or other Birna viruses. Also the use of cDNA of other Birna viruses (e.g. DXV, IPNV, OV or TV) leads to new IBDV vaccines. In this approach, one or more of the IBDV immunodominant or neutralizing epitopes are exchanged with the corresponding parts of the protein of another Birna virus.

Of course, the invention also provides a method for producing an rIBDV according to the invention, said vector comprising heterologous nucleic acid sequences derived from another virus, or (micro)organism, whereby r- or mIBDV serves as a vector. For example a method is provided to generate an infectious copy IBDV which expresses one or more antigens from other pathogens and which can be use to vaccinate against multiple diseases. Such an infectious copy IBDV for example comprises a heterologous cDNA encoding a heterologous protein obtained from a pathogen, for example poultry pathogens. Also a method is provided to generate a conditional lethal IBDV deletion mutant which can be used as self-restricted non-transmissible (carrier) vaccine. Such an IBDV deletion mutant is unable to express one of the IBDV proteins, and is phenotypically complemented by supplying the missing protein by other means.

The invention is further explained in the detailed description without limiting the invention thereto.

Detailed description

Material and Methods

5 Viruses and cells

The IBDV isolate CEF94 is a derivative of PV1 (Petek et al., 1973). After receiving the PV1 isolate in our laboratory in 1973, we have further adapted this isolated
10 by repeated passage (> 25 times) on either primary Chicken Embryo Fibroblast (CEF) cells or Bursa cells. The very virulent IBDV isolate D6948 was originally isolated in 1989 by the Poultry Health Service (Doorn, the Netherlands). It was purified by 5 passages in
15 embryonated eggs and one subsequent passage in SPF leghorn chickens. IBDV Serotype II isolate TY89 (McFerran et al., 1980) was maintained in our laboratory by a limited number of passages on CEF cells. Amplification of CEF-adapted isolates of IBDV (CEF94 and TY89) was
20 performed by growing freshly prepared chicken embryo fibroblast (CEF) cells in a tissue culture flask (75 cm²) until near confluency. This cell culture was infected with either CEF94 or TY89 (moi = 0.1) and incubated for 48 h at 37° C in a 5% CO₂ incubator. The supernatant of
25 this culture was centrifuged at 6000 g for 15 min. (pelleting of debris), transferred to clean tubes and subsequently centrifuged at 33.000 g for 3 h. The virus pellet was resuspended in PBS (1% of the initial culture volume). The very virulent IBDV isolate D6948 was
30 propagated in our laboratory in 21-days-old chickens by inoculation of 200 ELD50 (Egg Lethal Doses) per chicken, nasally and by eye-drop. The bursas of Fabricius were collected from the infected chickens three days post infection, and two volumes of tryptose phosphate buffer
35 was added. This mixture was homogenized in a Sorval Omnimixer (3 * 10 sec, maximum speed) and subsequently clarified by centrifugation (6000 g, 10 min). The supernatant was transferred to clean tubes and extracted

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three times with freon and once with chloroform. Virus preparations were stored at -70°C until further use. QM5 cells (Antin and Ordahl, 1991) were received from the laboratory of R. Duncan (Dalhousie University, Halifax, Nova Scotia, Canada) and maintained by using QT35 medium (Fort Dodge), in a CO_2 incubator (37°C).

Isolation of viral dsRNA

- 10 The genomic dsRNA was purified from the IBDV particles by digesting the viral proteins with Proteinase K (Amresco, 1.0 mg/ml) in the presence of 0.5 % SDS during 2 h at 50°C . The viral dsRNA was purified by phenol/chloroform/isoamylalcohol (25:24:1) extraction
- 15 (two times) and precipitation with ethanol (2.5V) / NaCl (0.1V, 5M, pH4.8) or with 2 M lithiumchloride (Diaz-Ruiz and Kaper, 1978). The RNA was dissolved in DEPC treated water (10 % of the initial volume) and stored at -20°C until further use. The integrity and purity of the viral
- 20 RNA was checked on an agarose gel.

Rapid Amplification of cDNA ends

- The extreme 5'-termini of all genomic RNA strands (the coding and non-coding strands of both the A- and B-segment) of isolate CEF94 were determined. We used 2 ug of genomic dsRNA and 10 pmol of strand- and segment specific primers in a total volume of 12 ul, for each determination. After incubation at 95°C for five min. we
- 25 transferred this mixture onto ice and added 4 ul of Superscript II first strand syntheses buffer (Gibco/BRL), 2 ul of 100 mM DTT and 2 ul of dNTP's (10 mM each). This mixture was subsequently incubated at 52°C for 2 min, after which 1 ul of reverse transcriptase (Superscript
- 30 II, Gibco/BRL) was added and incubation at 52°C was continued for one hour. The reverse transcriptase was inactivated by the addition of 1 ul of 0.5 M EDTA. The
- 35

genomic dsRNA was destroyed by the addition of 2 ul of 6 M NaOH and incubation at 65° C for 30 min. For neutralization, 2 ul of 6 M Acetic acid was added, and cDNA was recovered by using a Qiaex DNA extraction kit (Qiagen) and finally dissolved in 6 ul water. In the anchor ligation reaction we used 2.5 ul of the cDNA preparation, 4 pmol of the anchor, 5 ul T4 ligation buffer and 0.5 ul T4 RNA ligase (New England Biolabs). Incubation was performed at room temperature for 16 h and the reaction was stored at - 20° C. To amplify the single stranded cDNA which was ligated to the anchor, we used a nested primer in combination with the anchor primer. The PCR was performed by using the following conditions: 10 pmol of each specific primer, 10 pmol of the anchor primer, 4.5 or 5.5 mM MgCl₂, 1* Taq buffer (Perkin Elmer), 50 uM of each dNTP, 3 units of Taq polymerase (Perkin/Elmer), and 4 ul of the RNA ligation mixture as template, in a total volume of 50 ul. The amplification was performed by 35 cycles through the temperature levels of 92° C (45 sec), 57 or 65° C (45 sec), and 72° C (90 sec). The resulting PCR products were agarose gel purified and digested with EcoRI and XbaI and ligated (T4 DNA Ligase, Pharmacia), in a pUC18 vector which had previously been digested with the same restriction enzymes. The resulting plasmids were amplified in *E. coli* and nucleotide sequence analysis was performed by using the M13F and M13R primers.

Generation of full length A- and B-segment single stranded cDNA

To produce full length single stranded cDNA of both the A- and B-segments of CEF94 and D6948, we used a primer specific for the 3'-terminus of the coding strand in the reverse transcription reaction for initiation of the cDNA synthesis. As template we used 1 ug of genomic RNA and 2.5 pmol of ANC1 (A-segment specific primer, Table 2) or BNC1 (B-segment specific primer, Table 2) in

a total volume of 10 ul. After incubation at 98° C for two min. we transferred this mixture immediately onto ice and added 10 ul of RT-mix containing 2* Superscript II first strand syntheses buffer (Gibco/BRL), 20 mM DTT, 2 mM of each dNTP and 100 units of Superscript II (Gibco/BRL). In case of the negative control reaction the addition of Superscript II enzyme was omitted. All tubes were incubated at 50° C for 30 min, after which time 0.5 units of RNase H were added and incubation was continued at 37° C for 15 min. Water (80 ul) was added to each tube, and dsRNA and cDNA was purified by a phenol/chloroform/isoamylalcohol (25:24:1) extraction and precipitated by using a standard ethanol/NaAc precipitation protocol. Obtained pellets were dissolved in 20 ul of water and stored at -20° C.

Amplification of full length cDNA using a PCR based protocol

The full length single stranded cDNA of both the A- and B-segment were amplified by using PCR. The primers which hybridize to the 3'-terminus of the non-coding strand of the A-segment (T7AC0, Table 2) and B-segment (T7BC1, Table 2) both have a non-hybridizing 5' extension of 24 nt containing a T7 promoter sequence and an *EcoRI* site. The primers that hybridize to the 5' terminus of the coding strand of the A-segment (ANC0, Table 2) and B-segment (BNC1, Table 2) match exactly. As template we used 5 ul of the above mentioned RT reaction and the PCR was performed in the presence of 1* Expand High Fidelity buffer, 50 uM of each dNTP, 0.2 pmol of each primer, 1.5 units of Expand High Fidelity enzyme, and 2.0 mM MgCl₂ (A-segment) or 4.0 mM MgCl₂ (B-segment). Amplification was performed by cycling 35 times between 94° C (15 sec), 58° C (15 sec) and 72° C (5 min) in case of A-segment amplification (A-program) and cycling for 35 times between 94° C (15 sec), 54° C (15 sec) and 72° C (5 min)

in cases of B-segment amplification (B-program), using a Biometra T3 thermocycler. The yield of PCR products was checked on a 1.0% agarose gel.

5 Cloning and analysis of the generated PCR fragments

The full length PCR fragments which were generated three times independently from genomic dsRNA, were isolated from the agarose gel by using a Qiaex gel purification kit (Qiagen) and ligated in the pGEM-Teasy (Promega) vector according to the suppliers instructions. The ligated plasmids were used to transform *E. coli* DH5-alpha cells which were subsequently grown under ampicillin selection (100 ug/ml) and in the presence of IPTG (0.8 mg per petri-dish)) and Bluo-gal (0.8 mg per petri-dish). Plasmid DNA of white colonies was prepared and analyzed by restriction enzyme digestion and agarose gel separation. The nucleotide sequences of the cloned cDNA's were determined by using a ABI310 automated sequencer and A- and B-segment specific primers. The consensus nucleotide sequences of both segments of CEF94 and of both segments of D6948 were determined (Fig. 2) and the corresponding amino acid sequence of the open reading frames was deduced (Fig. 3). By using the cDNA of two independent clones we restored one amino acid mutation present in the A-segment clone (V542A), resulting in pHB-36W, one amino acid mutation in the A-segment clone of D6948 (P677L), resulting in pHB-60, and one amino acid mutation in the B-segment of D6948 (Q291X), resulting in pHB-55. No amino acid mutations were present in the B-segment cDNA clone of CEF94 (pHB-34Z).

Introduction of a Hepatitis Delta Virus ribozym

35 The Hepatitis Delta Virus ribozym was first introduced into the *E. coli* high copy number plasmid pUC-18 by digesting transcription vector 2.0 (Pattnaik et al., 1992) with restriction enzymes *Xba*I and *Sma*I. The

resulting 236 bp fragment, which contains the Hepatitis Delta Virus Ribozyme and a T7 RNA polymerase terminator, was ligated in the pUC18 vector which previously was digested with *Xba*I and *Sma*I, yielding pUC-Ribo. Plasmids containing the A- and B-segment of CEF94 and D6948 were used as template in a full length PCR using the above described conditions, and primers specific for either the A-segment (T7AC0 and ANC0) and B-segment (T7BC1 and BNC1). The PCR fragments were agarose gel purified (Qiaex), blunt-ended by using T4 DNA polymerase, and subsequently digested with *Eco*RI. The resulting DNA fragments were directionally cloned into the pUC-Ribo vector which previously had been digested with *Sma*I and *Eco*RI. The resulting plasmids were used as template in an *in vitro* transcription-translation reaction (TnT-T7Quick, Promega). The autoradiogram of SDS-PAGE analyses of the translation products revealed three dominant bands pVP2 (48-49 kDa), VP3 (32-33 kDa), and VP4 (28-29 kDa) when pHB-36A (A-segment of CEF94) or pHB-60 (A-segment of D6948) was used as template. One dominant band (VP1 (91 kDa)) was found when we used plasmid pHB-34Z (B-segment of CEF94) or pHB-55 (B-segment of D6948) as template (data not shown).

25 Introduction of a genetic tag

To distinguish infectious virus generated from cloned cDNA from wild-type virus we introduced a genetic tag in the 3'-UTR of the A-segment of IBDV-A isolate. Two nucleotides of pHB-36A were mutated (C3172T and 3T173A) thereby introducing a unique *Kpn*I restriction site (GGTAAC). These mutations were introduced by the method described by Higuchi (1990). A 383 bp fragment of the resulting PCR fragment was ligated (Rapid ligation kit, Boehringer Mannheim) into the full length A-segment clone (pHB-36A) by using two unique restriction sites (*Bgl*III and *B*lpI). The resulting plasmid pHB-36W was amplified in *E. coli*. The genetic tag was present in this

full length CEF94 A-segment clone, as could be concluded from sequence analysis and digestion with restriction enzyme *KpnI* (data not shown). No difference was observed in the resulting protein pattern when either pHB-36A or
5 pHB-36W was used as template in an *in vitro* transcription/translation reaction (data not shown).

Construction of mosaic A-segment cDNA

10 We constructed plasmids containing mosaic IBDV A-segments which partly consisted of cDNA of one isolate (CEF94) and partly of cDNA of another isolate (D6948). To construct these plasmids we have amplified specific parts of cDNA using appropriate IBDV specific or selective primers. The
15 amplified PCR fragment of cDNA of D6948 was subsequently used to replace the corresponding part in plasmids pHB-36W, using restriction endonucleases and T4 DNA ligase (Rapid DNA Ligation, Boehringer Mannheim).

For the construction of pHB36-vvVP2 (exchange of
20 pVP2 encoding part, Table 4) we have used IBDV specific to generate the mosaic PCR-VP2D fragment (2256 bp, see Fig. 5a). The internal part of this PCR fragment was used to exchange the corresponding part of pHB-36W, using unique sites for restriction enzymes *EcoRI* and *SacII*.

25 For the construction of plasmid pHB36-vvVP3 (Table 4) we used IBDV specific primers to generate a mosaic PCR-VP3c fragment (2154 bp, see Fig. 5b). The internal part of this PCR fragment was used to exchange the corresponding part of pHB-36W, using unique sites for the *EagI* and *KpnI*
30 (genetic tag site) restriction enzymes.

For the construction of plasmid pHB36-vvVP4 (Table 4) we used IBDV specific primers to generate a mosaic PCR-VP4d fragment (2154 bp, see Fig. 5c). The internal part of this PCR fragment was used to exchange the corresponding
35 part of pHB-36W, using the unique site for restriction enzymes for *EagI* and *DraIII*.

plasmids pHB36-vvVP2, -vvVP3, and -vvVP4 were partly analysed by nucleotide sequence determination to conform

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that no unintended mutations were introduced during the described manipulations.

Introduction of a serological marker

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To obtain the cDNA of the A-segment of a serotype II IBDV isolate we generated single stranded cDNA of TY89 as described above, by using the ANC1 primer. The coding region of the VP3 protein was subsequently three times
10 independently amplified in a PCR by using 2 ml of RT-material, 1* Taq buffer, 50 uM of each dNTP, two IBDV serotype II specific primers (0.2 pMol each), 1.5 units of enzyme, and 3.0 mM MgCl₂ in a 0.1 ml reaction volume. Amplification was performed by cycling 35 times between
15 94° C (15 sec), 52° C (15 sec) and 72° C (1 min). The resulting 956 bp fragment was cloned in the pGEM-TEasy vector and the consensus nucleotide sequence was determined (Fig. 2a). One of the isolated plasmids contained the TY89 VP3 consensus sequence (pSV-VP3-TY89,
20 Fig. 4) and was used as template to generate a 893 bp PCR fragment (see Fig. 5d). This PCR fragment was subsequently used to replace the corresponding part of plasmid pHB36W-vvVP3, by using the artificially introduced KpnI (nt 3175) and SacII (nt 1760) restriction
25 sites in both plasmid pSV-VP3-TY89 and pHB36W-vvVP3. The resulting plasmid (pHB36-vvVP3, see Fig. 5d) encodes the N-terminal 722 amino acids of the CEF94 polyprotein and the 290 C-terminal amino acids of the TY89 polyprotein. The intended exchange was confirmed by nucleotide
30 sequence analysis.

The same approach was used to exchange the C-terminal half of the coding sequence of the VP3 protein. In stead of the artificially introduced SacII site, we made use of
35 the ScaI (nt 2799) site which is naturally present both in the TY89 and in the CEF94 cDNA of the A-segment, in combination with the artificially introduced KpnI site (nt 3172). The resulting plasmid (pHB36-s2VP3C, Table 4)

encodes a polyprotein consisting of the N-terminal 890 amino acid of the CEF94 polyprotein, in combination with the C-terminal 122 amino acids of the TY89 polyprotein.

- 5 For the construction of plasmid pHB36-s2VP3N (see Table 4) we have replaced the ScaI (nt 2799) - KpnI (nt 3172) part of plasmid pHB36-s2VP3 with the corresponding part of plasmid pHB-36W. Using the specific restriction endonucleases ScaI and KpnI, and T4 ligase. The
- 10 nucleotide sequence of plasmid pHB36-s2VP3N was conformed by sequence analysis.

- For the introduction of the C-terminal encoding part of the VP3 protein of IBDV isolate TY89 into the cDNA of
- 15 isolate D6948 we have exchanged part of plasmid pHB-60 (nt 1760 -> nt 3260) with the corresponding part of plasmid pHB36-s2VP3C. Plasmid pHB36-s2VP3C was digested with restriction enzymes SacII and XbaI and a 1735 bp fragment was recovered from an agarose gel by Qiaex gel
- 20 extraction kit (Qiaex). This DNA fragment was ligated in the 4440 bp vector fragment of pHB-60 which had previously been digested with the same restriction enzymes. The resulting plasmid (pHB60-s2VP3C1, Table 4) contains cDNA derived from IBDV isolate D6948 (nt 1 to
- 25 1760), CEF94 (nt 1760 to 2799 and nt 3175 to 3260), and TY89 (nt 2799 to 3175).

- 30 Transfection of QM5 cells

- QM5 cells, grown to 80% confluency in 60 mm dishes, were infected with Fowl Pox T7 (FPT7) (Britton et al., 1996) one hour prior to transfection. FPT7 infected QM5
- 35 cells were subsequently washed once with 5 ml QT-35 medium and incubated with 2 ml fresh Optimem 1 (Gibco/BRL) two times during 15 min. In the mean time, DNA (2.0 to 4.0 ug) was incubated in 0.5 ml Optimem 1

supplemented with 25 μ l LipofectAMINE (Gibco/BRL) and kept at room temperature for at least 30 min. The washed QM5 cells were covered with 4 ml of Optimem 1, the DNA/LipofectAMINE transfection mixture was added and the cells were stored for 18h in a 5.0% CO₂ incubator at 37°C.

Detection of recombinant IBDV after transfection of QM5 cells

10

Transfected QM5 cells were washed once with PBS after the transfection. Infectious recombinant IBDV (rIBDV) was recovered from transfected QM5 cells by covering them with 4 ml of QT-35 medium supplemented with 5% fetal calf serum and 2% of an antibiotic mix (1000 U/ml Penicillin, 1000 μ g/ml Streptomycin, 20 μ g/ml Fungizone, 500 μ g/ml; Polymixin B, and 10 mg/ml Kanamycin) and incubation for 24 h at 37°C (5.0% CO₂). The supernatant was filtered through a 200 mM filter (Acrodisc) to remove FPT7 virus

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and was subsequently stored at -70°C or used directly for quantitation of rIBDV. Recombinant mosaic IBDV (mIBDV) which contains at least the pVP2 from vvIBDV isolate D6948 is unable to re-infect QM5 cells (see Table 5). Therefore, supernatant of transfection experiments

25

which contained D6948 pVP2 encoding cDNA were used to infect 11-days-old, embryonated eggs via the chorioallantoic membrane (CAM) route. To determine the presence of infectious IBDV, the embryo's were collected five days post-infection, homogenized by using a Sorval Omnimixer (3 * 10 sec, max. speed) and assayed for the presence of IBDV proteins in a IBDV protein specific Elisa.

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Serological differentiation of recombinant mosaic IBDV (mIBDV)

Different monoclonal antibodies were used to detect
5 recombinant mosaic IBDV (mIBDV) that contained part of
the TY89 VP3 or the complete TY89 VP3. The mIBDV's were
used to infect QM5 or primary bursa cells and incubated
for 24h (QM5 cells) or 48h (primary bursa cells) in a 5%
CO₂ incubator at 37° C or 39° C, respectively. The
10 infected cells were subsequently fixed and an
immunoperoxidase monolayer assay (IPMA) was performed by
using monoclonal antibodies which are either specific for
VP2 of serotype I IBDV (Mab 1.4), or specific for VP3 of
serotype II (Mab T75), or specific for VP3 of serotype I
15 (Mab B-10 or C-3).

Virulence of rIBDV in young SPF chickens

To evaluate the degree of virulence of the generated
20 rIBDV, srIBDV, and mIBDV isolates we have inoculated 12
groups (10 21-days old SPF chickens) with these viruses.
Each chicken received nasally and by eye-drop 1000 ELD50
IBDV, with exception with the negative control group,
which received only PBS. The animals were monitored for
25 clinical signs and dead chicks were removed each day. At
9 days post infection, all the chicks from the negative
control groups and all the surviving chicks from groups
in which mortality had occurred, were bled (5 ml) and
euthanized for necropsy. From the other groups, 6 chicks
30 were bled (5 ml) and taken for necropsy at day 9 post
infection, where as the remaining 4 were bled (5 ml) and
taken for necropsy at day 15 post infection. Bursa and
body weight was determined of all chicks which had been
euthanized at day 9 post infection

Results

Nucleotide sequence determination of the 5'-termini.

- 5 One group has reported the 5'- and 3'-terminal sequences of the segmented dsRNA genome of IBDV (Mundt and Muller, 1995). To verify the terminal sequence of the genome of IBDV and to be able to produce the exact cDNA sequence of a single IBDV isolate we have determined the 5' terminal
- 10 sequences of both the coding and non-coding strands of the two genomic segments of CEF94, a Chicken Embryo Fibroblast (CEF) adapted, classical isolate of IBDV, by using the RACE (Rapid Amplification of cDNA Ends) technique (Frohman et al., 1988). The RACE analysis was
- 15 performed in duplicate on each of the four 5'-termini of the CEF94 genome. The resulting sequence data (Table 3) show that the length of the 5'-termini of the coding strands was the same in all cases. Furthermore we found that the nucleotide sequence was identical, except for
- 20 the last nucleotide which varied in a few clones. This is in contrast to the sequence data of the 5'-termini of the non-coding strands, which varied in length considerably. We also found that the last nucleotide, although preferably a cytosine, varied in some clones similarly to
- 25 what we found for the 5'-termini of the coding strands. The consensus sequence for the 3'-terminal nucleotide of the A-segment coding strand of CEF94 differs from the nucleotide sequence reported by Mundt and Muller (Mundt and Muller, 1995), i.e. being a cytosine in stead of a
- 30 thymine.

Generation of plasmids containing full length IBDV cDNA

- 35 Using the sequence data of the 5'-termini we cloned the entire coding and non-coding cDNA sequences of the A-segment and B-segment of classical isolate CEF94 by means of RT-PCR. Using the same procedure and using the same primers we also generated the entire coding and non-coding cDNA of the A- and B-segment of a non-CEF-adapted,

very virulent IBDV isolate D6948. The nucleotide sequence of the entire genome of both isolates was determined three times independently. This sequence information enabled us to generate a consensus nucleotide sequence of both the A- and B-segments of IBDV isolates CEF94 and D6948 (Fig. 2A).

Fowlpox T7 polymerase expression system

One system for generating infectious IBDV virus using *in vitro* synthesized mRNA derived from cDNA of a CEF-adapted IBDV isolate has previously been described (Mundt and Vakharia, 1996). This system is based upon *in vitro* run-off transcription from the T7 promoter which was artificially introduced in front of the cDNA sequences of the A- and B-segments. This RNA is subsequently transfected into VERO cells, after which infectious IBDV virus could be harvested from these cells. One of the drawbacks of this system is that the *in vitro* generated RNA has to contain a 3'-G-ppp5'-(cap structure) on its 5'-end in order to get translation of the introduced RNA into the viral proteins, and hence replication of viral RNA. The *in vitro* production of high quality mRNA is both inefficient and expensive as a cap structure has to be present at the 5'-end. Furthermore, expression levels from transfected RNA are generally low due to the short half-life of RNA. To circumvent the drawbacks of generating *in vitro* capped RNA and low expression levels, we have explored the possibility of using an *in vivo* based T7-expression system (Fowlpox T7 polymerase expression system, (Britton et al., 1996) for generation of viral RNA from plasmids containing full length IBDV cDNA.

Generating of infectious IBDV using Fowlpox infected cells

To be able to generate IBDV from cloned cDNA which
5 has the authentic terminal sequences, we introduced the
cis-acting Hepatitis Delta Virus (HDV) ribozym (Chowrira
et al., 1994) downstream of the cDNA sequence of the A-
and B-segments (Fig. 4). Furthermore we introduced an
additional modification in 3' untranslated region of the
10 CEF94 A-segment. By exchanging 2 nucleotides we
introduced a unique KpnI endonuclease restriction site in
this cDNA. The introduction of this unique restriction
site enables us to distinguish between wild-type IBDV and
infectious IBDV virus generated from cloned cDNA
15 (genetically tagged rIBDV). As expected, this plasmid
yields the same viral proteins in an *in vitro*
transcription-translation reaction as the A-segment clone
without the genetic tag (data not shown).
Plasmid pHB-36W (A-segment CEF94), pHB-60 (A-segment
20 D6948), pHB-34Z (B-segment CEF94), and pHB-55 (B-segment
D6948) were used individually to transfect FPT7 infected
QM5 cells as described in the Materials and Method
section. To analyze whether the transfected QM5 cells
expressed IBDV proteins, we performed an IPMA, 24 h after
25 transfection. We used polyclonal antiserum directed
either against VP3 (pHB-36W and pHB-60 transfections) or
VP1 (pHB-34Z and pHB-55 transfections) in this analysis.
About 10 to 50% of the QM5 cell expressed VP3 after
transfection with pHB-36W or pHB-60 (data not shown).
30 When B-segment encoding plasmids were used (pHB-34Z or
pHB-55) we found that the same percentage of cells (about
10 to 50%) were expressing VP1 (data not shown).
Subsequently, we co-transfected combinations of plasmids
containing the A- and B-segment cDNA's into FPT7 infected
35 QM5 cells. To screen for infectious recombinant IBDV
(rIBDV) in the supernatant of the transfected QM5 cells,
we transferred part of the supernatant (10% of the
volume) after 18 h onto fresh QM5 cells or onto primary

bursa cells. We only could detect rIBDV when A-segments plasmids in combination with B-segments plasmids were used to transfect the QM5 cells. rIBDV could not be detected when supernatant of the cells transfected with

5 A-segment (pHB-60) and B-segment (pHB-55) of D6948 (rD6948) was transferred onto QM5 cells. However, when the co-transfection supernatant of pHB-60 and pHB-55 was transferred onto primary bursa cells or embryonated eggs we were able to show the presence of infectious IBDV

10 (rD6948) in primary bursa cells (after 48h) and in embryonated eggs (after five days). The presence of rIBDV in the first passage was established by using either an IPMA (QM5 cells or primary bursa cells) or an IBDV specific Elisa (embryonated eggs). The generated rCEF94

15 and rD6948 isolates were amplified in 10-days old embryonated SPF eggs and subsequently used to infect 21-days old SPF chickens (10 chickens per IBDV isolate). The resulting data of the animal experiment (Table 6) shows that the mortality, body weight, bursa weight, and bursa-

20 body weight ratio, caused by rD6948 are the same as the parent very virulent D6948 isolate. Also at necropsy, gross lesions of bursa were as severe for rD6948 as for the parental D6948 isolate (data not shown). From this chicken experiment it is concluded that rD6948 has

25 retained the properties of a very virulent IBDV isolate, and is truly a very virulent rIBDV.

Detection of the genetic tag

30 Supernatant of rCEF94 infected QM5 cells was harvested and IBDV was isolated by centrifugation as described in the material section. The dsRNA genome was extracted and an A-segment specific primer was used to generate single stranded cDNA, by using reverse transcriptase. The cDNA

35 was subsequently amplified by PCR. The generated PCR fragment was cloned into a high copy number *E. coli* plasmid (pGEM-Teasy, Promega) and was either digested with *KpnI* or used for nucleotide sequence determination.

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The presence of the genetic tag in rCEF94 was confirmed in both analyses.

Identification of a lethal amino acid mutation in VP4

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Plasmid pHB-36 (A-segment CEF94, Table 4) contained a single nucleotide substitution at position 1875 (thymine in stead of a cytosine) compared to the consensus CEF94 A-segment sequence (Fig. 2A). This nucleotide

10 substitution leads to a valine at position 582 of the polyprotein in stead of an alanine, which is encoded by the consensus sequence (V582A, Fig. 3A). As this amino acid mutation is present in the viral protease (VP4), we subsequently checked whether this protease was still able
15 to autocatalytically liberate the viral proteins (pVP2, VP3 en VP4) from the polyprotein. When plasmid pHB-36 was used as template in a coupled in vitro

transcription/translation reaction in the presence of ³⁵S labeled methionine we found a delayed splicing of the
20 polyprotein (data not shown). Apart from the viral proteins which are found in case of normally spliced polyprotein (pVP2, VP3 and VP4), we found intermediate spliced products (60 kDa: VP4+VP3), and non-spliced polyprotein (data not shown). Although the viral protease

25 (VP4) of clone pHB-36 is able to liberate the structural viral proteins (pVP2 and VP3) from the polyprotein, this clone did not yield rIBDV when using the FPT7 based transfection protocol as described above. Rapid

autocatalytic cleavage of the polyprotein is apparently
30 necessary for the generation of infectious rIBDV. We expect that other mutations within VP4 which alter the rate or specificity of the autocatalytic cleavage of the polyprotein will also have a negative effect on viability of the generated rIBDV. Furthermore mutations in the

35 region of the cleavage sites (pVP2-VP4 and VP4-VP3) may also have a negative effect on replication of rIBDV. Any mutation, introduced by modern molecular biological techniques into the cDNA of a very virulent IBDV may

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enable us to generate rIBDV which has a reduced viability and which can be used as a live or killed IBDV vaccine.

Generation of segment reassortant IBDV

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Transfection of CEF94 A-segment cDNA (pHB-36W) in combination with D6948 B-segment cDNA (pHB-55) yielded segment reassorted IBDV (srIBDV-CADB) when supernatant of QM5 transfected cells was transferred onto fresh QM5
10 cells (Table 5). When D6948 A-segment cDNA (pHB-60) was used in combination with CEF94 B-segment cDNA (pHB-34Z) no infectious srIBDV (srIBDV-DACB) could be detected on QM5 cells (Table 5). However, when primary bursa cells were used to assay for the presence of infectious IBDV we
15 found in both cases (srIBDV-CADB and srIBDV-DACB) infected cells after 24h of incubation. Out of the population of primary bursa cells, only lymphoid cells were infected with srIBDV-DACB, while both lymphoid and fibroblast cells were infected in the case of srIBDV-
20 CADB. The srIBDV-DACB isolate induces the same clinical signs as D6948, while the srIBDV-CADB isolate has a comparable virulence as CEF94 (Table 6).

Construction of mosaic IBDV

25

By using modern molecular biological techniques such as those described above, we have created mosaic recombinant IBDV (mIBDV) which exists partly of cDNA derived from CEF94, and partly from D6948 (vvIBDV) or TY89 (a serotype
30 II IBDV isolate). Replacement of the pVP2 protein encoding sequence of CEF94 by the corresponding part of the D6948 yielded only virus (mCEF94-vvVP2) when the supernatant of transfected cells was transferred to cells which are normally susceptible for non CEF-adapted
35 vvIBDV, i.e. primary bursa cells or embryonated eggs. (Table 5). Replacement of the VP3 or VP4 protein encoding sequence of CEF94 with the corresponding part of D6948 yielded mIBDV by using QM5 cells as recipient in the first passage (mCEF-vvVP3 and mCEF-vvVP4 respectively).

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Replacement of the complete VP3 cDNA (290 amino acids) of CEF94 by the corresponding part of the TY89 cDNA yielded a plasmid which encoded a polyprotein consisting of pVP2 and VP4 derived from CEF94 and of VP3 derived from TY89. When this plasmid (pHB36-s2VP3) was used in an in vitro transcription-translation reaction, all the expected proteins, pVP2, VP4 and VP3 were present (data not shown). Transfection of this plasmid in combination with a plasmid (pHB-34Z) containing the B-segment cDNA of CEF94 yielded a mosaic IBDV (mCEF94-s2VP3). Two monoclonal antibodies which are specific for serotype I VP3 (Mab B10 and C3) were unable to recognize this mCEF94-s2VP3, while an antibody which is specific for the serotype II VP3 (Mab T75) did recognize this mosaic virus (Table 5). As expected the mCEF94-s2VP3 was also recognized by a serotype I specific, neutralizing monoclonal antibody directed against VP2 of the CEF94 isolate (Mab 1.4). The TCID₅₀ on QM5 cells, which was determined 18 hours after transfection, was considerably lower (3 logs) in the case of mCEF94-s2VP3 compared to rCEF94. Furthermore we found that only single QM5 cells were infected by mCEF94-s2VP3 after 24 h. This is in contrast to the plaque forming phenotype of CEF94 and rCEF94 on QM5 cells after 24 h of infection. To generate mIBDV which has the same replication and plaque forming characteristics as rCEF94, but which is still antigenetically different from rCEF94 we subsequently exchanged only the N-terminal part (168 amino acids) or C-terminal part (122 amino acids) of the VP3 of CEF94 by the corresponding sequence of TY89. When these mosaic A-segment plasmid (pHB36-s2VP3N or pHB36-s2VP3C) were transfected in combination with pHB-34Z (CEF94 B-segment) we obtained mosaic IBDVs (mCEF94-s2VP3N or mCEF94-s2VP3C) with replication capabilities in QM5 cells that are equal (mCEF94-s2VP3C) or slightly reduced (mCEF94-s2VP3N) to those of rCEF94 IBDV (data not shown).

Subsequently we checked the recognition of mCEF94-s2VP3N and mCEF94-s2VP3C virus by several Mabs in an IPMA on QM5 infected cells (Table 5). Mab T75 which is specific for VP3 of serotype II also recognized mCEF94-s2VP3C, while
5 the recognition of mCEF94-s2VP3N was slightly reduced. Mab B10, which is specific for VP3 of serotype I did not recognize rCEF94-s2VP3C, while it still recognized mCEF94-s2VP3N. Another Mab (C3) which did not react with mCEF94-s2VP3 infected cells did react with mCEF94-s2VP3C
10 infected cells, although the reaction was reduced compared to QM5 cells infected with rCEF94 (Table 5) mCEF94-s2VP3N was not recognized by Mab C3. The serotype I specific, neutralizing antibody Mab 1.4 which recognizes VP2 recognized, as expected, both mCEF94-s2VP3N and mCEF94-s2VP3C.
15

The coding sequence of the C-terminal part of serotype II VP3 (122 amino acids) was also used to replace the corresponding part of the cDNA of D6948. During the exchange we have replaced some D6948 cDNA
20 sequence (encoding for C-terminal part of VP4 and the N-terminal part of VP3, and the 3'-UTR) with the corresponding sequence of CEF-94 (see Fig. 5g). The resulting plasmid (pHB60-s2VP3C1) was, together with pHB-55 (B-segment D6948), transfected into FPT7 infected QM5
25 cells. Supernatant of these transfected QM5 cells was collected after 24 h and was transferred to embryonated eggs and primary bursa cells. By using monoclonal antibodies we were able to detect infected cells in the monolayer of primary bursa derived cells (see Table 5).
30 mD6948-s2VP3C1 gave the same reaction pattern with the monoclonal antibodies as mCEF-s2VP3C did. Isolate mD6948-s2VP3C1 (1000 ELD50/chicken) was also used to infect 10 SPF chickens (21-days old) to evaluate its virulence. This mIBDV isolate did not cause any mortality in a 9-
35 days course, opposite to the D6948, rD6948 and srIBDV-DACB isolates (Table 6). However, the bursa is severely damaged by this mIBDV, as the bursa-body weight ratio of this group is same as found in the groups which received

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D6948 or rD6948. This indicates that mD6948-s2VP3C1 is still able to replicate and induce apoptosis in the bursa of Fabricius.

5 Example 1

10 Infectious Bursal Disease Virus is the causative agent of one of the most important and wide-spread infectious diseases among commercial chicken flocks. IBDV causes a depletion of B-lymphoid cells in the bursa of Fabricius, inducing immunosuppression, morbidity, or even acute mortality. Because currently used live IBDV vaccines are derivatives from field isolates no serologic discrimination between field isolates and live vaccines can be made. The recently developed reverse
15 genetics techniques for IBDV allows one to generating genetically modified IBDVs which might have altered biological and antigenic properties. Here, we describe the rescue of mosaic serotype I IBDVs, where the polyprotein encoding region was partly replaced by the corresponding region of a serotype
20 II strain. A mosaic virus, containing the C-terminal part of serotype II VP3 showed only a slightly delayed release of progeny virus compared to unmodified serotype I virus, while maximum viral titers at 25h post infection were equal. Since serotype specific epitope(s) are present in the C-terminal part
25 of VP3, we were able to discriminate this rescued virus from serotype I and II IBDV strains. These findings makes the use of a chimeric VP3 a promising approach to develop an IBDV marker vaccine.

30 Infectious Bursal Disease Virus (IBDV), a member of the Birnaviridae, is the causative agent of a highly contagious disease of chickens. This disease is also known as Gumboro, named after the place where the first outbreak of IBDV was reported (Cosgrove, 1962). The double stranded RNA (dsRNA) genome, which is divided over two segments (Dobos et al.,
35 1979), is packed within a capsid, consisting of two viral proteins (VP2 and VP3). The resulting single-shelled naked virus particle has a diameter of 60 nm, and an icosahedral (T=13) symmetry (Bottcher et al., 1997).

The largest dsRNA segment (A-segment, 3260 bp) contains two partly overlapping open reading frames (ORFs). The first, smallest ORF encodes the non-structural viral protein VP5 (145-149 amino acids, 17 kDa). The second ORF encodes a polyprotein (1012 amino acids, 110 kDa), which is rapidly cleaved into three proteins: pVP2 (48 kDa), VP4 (29 kDa) and VP3 (33 kDa) by the autocatalytic activity of VP4, both in vivo (Dobos et al., 1979) and in vitro (Hudson et al., 1986). During virus maturation pVP2 is further processed into VP2 (41-38 kDa), probably resulting from site-specific cleavage of pVP2 by a host cell encoded protease (Kibenge et al., 1997). The B-segment (2827 bp) contains one large ORF, encoding VP1 (877-881 amino acids, 91 kDa). This RNA dependent RNA polymerase is also a structural protein since it is found within the viral particle both as a free protein, and covalently linked to the 5'-ends of the genomic RNA strands (genome-linked protein, VPg) (Dobos, 1993; Spies and Muller, 1990).

Two different serotypes of IBDV have been described (serotype I and II) (McFerran et al., 1980). Wild-type serotype I IBDV strains have a specific tropism for developing B-lymphoid cells in the bursa of Fabricius. Serotype I viruses are sub-divided into classical strains, antigenic variant strains, and very virulent strains. Antigenic variant IBDV strains have only been reported in the USA (since 1985 (Snyder, 1990)) and were found to have a different pathologic phenotype. These antigenic variants appeared to have single amino acid changes in a specific region of the VP2 protein (the hypervariable region) that are probably responsible for the difference in phenotype. From 1988 on, European IBDV isolates were described that had an enhanced virulence (very virulent IBDV, vvIBDV), while having the same antigenic structure as classical isolates (Chettle, Stuart, and Wyeth, 1989). Although most nucleotide differences between classical and very virulent IBDV strains are found in the B-segment, we recently showed that the determinant(s) responsible for the enhanced virulence of a vvIBDV strain are located on the A-segment.

Unlike serotype I strains, wild-type serotype II strains do not have a specific tropism for B-lymphoid cells. Serotype II

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strains are naturally able to replicate in different tissues and can even be propagated on cell lines. Serotype II strains are usually recovered from turkeys and are neither pathogenic for turkeys (Jackwood et al., 1984) nor for chickens (Ismail, 5 Saif, and Moorhead, 1988). Conformation dependent, neutralizing epitopes are found within the hypervariable region of the capsid protein VP2 of both serotype I and II strains. Apart from VP2, the virion contains another abundant capsid protein, VP3. This protein does not contain virus-neutralizing epitopes, 10 although a fast immune response is found against linear VP3 epitopes both after vaccination and infection (Fahey, IJ, and Bagust, 1985). It has been shown that some of the epitopes present in VP3 of serotype I and II strains are recognized by the same monoclonal antibody (group-specific epitopes) while 15 other VP3 epitopes are serotype-specific (Mahardika and Becht, 1995; Oppling, Muller, and Brecht, 1991; Reddy, Silim, and Ratcliffe, 1992).

Differentiation between live serotype I IBDV vaccines and field isolates recovered during field outbreaks is difficult 20 and laborious as the genetic structure of the isolate in question has to be determined. Usually an RT-PCR is performed on the A-segment dsRNA, followed by sequence analysis (Eterradossi et al., 1999) or RFLP (Jackwood and Sommer, 1999). In search for an IBDV vaccine that can easily be distinguished 25 from IBDV field isolates by means of a serologic test, we set out to alter the antigenic structure of a serotype I IBDV strain. During the last few years several reports on the rescue of IBDV from cloned cDNA have been published (Boot et al., 1999; Lim et al., 1999; Mundt and Vakharia, 1996). Using our 30 reverse genetics system we have shown that we are able to generate virus from A-segment cDNA clones that are derived from dsRNA of different strains (i.e. classical adapted and a very virulent. In this report, we describe the rescue of a mosaic IBDV in which VP3 of a serotype I strain (CEF94) was (partly) 35 replaced by the corresponding part of a serotype II strain (TY89). Furthermore, we show that we are able to discriminate two of the rescued mosaic IBDV's from serotype I and II IBDV

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strains, by using a set of serotype specific monoclonal antibodies.

Methods

5

Viruses, cells, and antibodies. The classical IBDV serotype I strain CEF94 is a derivative of PV1 which has been adapted for growth on cell cultures. (Boot et al., 1999; Petek, D'Aprile, and Cancellotti, 1973). IBDV strain TY89 is the
10 prototype serotype II IBDV strain (McFerran et al., 1980) and is naturally able to grow on cell lines. Recombinant Fowlpox virus containing the T7 polymerase gene (FPV-T7) (Britton et al., 1996) was received from the laboratory of M. Skinner (Compton Laboratory, Berks, United Kingdom). QM5 cells, which
15 are susceptible for both TY89 and CEF94 and for FPV-T7, were grown in QT35 medium (Gibco/BRL), supplemented with 5% Fetal Calf Serum (FCS) and 2% antibiotics solution ABII (1000 U/ml Penicillin, 1000 mg/ml Streptomycin, 20 mg/ml Fungizone, 500 mg/ml Polymixin B, and 10 mg/ml Kanamycin), in a CO₂ (5%)
20 incubator at 37° C. Polyclonal antiserum against VP3/4 was produced by injecting rabbits with purified protein originating from bacteria transformed with an expression plasmid containing amino acids 722 to 1018 of the CEF94 polyprotein. Monoclonal antibodies IV, VII (Mahardika and Becht, 1995), and I/G4
25 (Oppling, Muller, and Brecht, 1991) were a kind gift of Dr. Müller, University of Leipzig (Germany). Mab 17/80 (Azad et al., 1986) was a kind gift of Dr. Lehrbach, Fort Dodge (Castle Hill, Australia). Monoclonal antibodies 1.4, 9.7, and 9.8 were prepared in our laboratory using standard procedures, and using
30 purified serotype I (CEF94) IBDV as antigen.

Generation and cloning of IBDV cDNA sequences. The generation of pUC19-based plasmids containing the full-length cDNA of the two genomic dsRNA segments of CEF94 has been
35 described before. The cDNA in these plasmids is preceded by a T7-promoter sequence and followed by the antigenomic Hepatitis Delta Virus ribozyme (Been, Perrotta, and Rosenstein, 1992) and

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a T7 terminator sequence. The entire VP3 encoding part of the serotype II strain TY89 was cloned by performing a RT-PCR on the dsRNA of TY89. The reverse transcription was performed with primer ANC1 (GGGGACCCGCGAACGG). Three independent PCRs were performed using primers RTAM (AATTGGTGTCCACACCTG) and RTAP (ATACAGGACCTAACTGGG) and 35 amplification cycles (annealing temperature of 52° C, and *Taq* polymerase). The resulting fragments (956 bp) were separately cloned into the pGEM-T Easy vector (Promega). The nucleotide sequence was determined on both strands using specific primers in a cycle sequencing reaction (BigDye terminator kit, PE Applied Biosystems) and an ABI310 DNA sequencer (PE Applied Biosystems). The consensus nucleotide sequence of the VP3 cDNA of TY89 was determined and the amino acid sequence (Fig. 6) was deduced. One of the clones, pSV-VP3-TY89(1), contained the consensus sequence and was used to generate the mosaic A-segment cDNA clones (see below).

Construction of the mosaic A-segment plasmid pHB36-s2VP3.

20 To replace VP3 of CEF94 with VP3 of TY89 in the full-length A-segment clone of CEF94, we amplified the TY89 VP3 encoding part by PCR using primers HY3P (AACGTTTTCTCACA TCCgCGgGACTGGG, non-hybridizing nucleotides are given as lowercase letters) and AGTM (GAGACTCCCAGGtaCCTCACTC) (annealing temperature is 58° C, *Pwo* polymerase, and 30 cycles). This PCR fragment (893 bp) was digested with *Sac*II (2316) and *Kpn*I (nt 3172), resulting in a 856 bp fragment, which was agarose gel purified (Qiaex gel extraction kit, Qiagen). The fragment was subsequently used to replace the corresponding part of plasmid pHB36-vvVP3, which had been digested with *Sac*II (partially, nt 2316) and *Kpn*I (nt 3172). After ligation of the PCR fragment into the vector (Rapid ligation Kit, Roche Molecular Biochemicals) we transformed *E. coli* DH5a cells with this mixture. Plasmids obtained from several independent colonies were analyzed and one plasmid having the intended sequence as determined by sequencing analysis was selected (pHB36-s2VP3, see Fig. 7).

Construction of mosaic VP3 cDNAs. To replace the N-terminal part of serotype I VP3, we used the same PCR as mentioned above (pHB36-s2VP3), only this time we used pHB-36W (A-segment CEF94) as a template, yielding a PCR fragment encoding the VP3

5 sequence of CEF94. This PCR fragment was digested with *ScaI* (nt 2799) and *KpnI* (nt 3172) to yield a 373 bp fragment. The agarose gel purified fragment was subsequently used to replace the corresponding part of pHB36-s2VP3, which had been digested partially with *ScaI* (nt 2799) and *KpnI* (nt 3172), yielding
10 pHB36-s2VP3N (see Table 9).

To replace the C-terminal part of serotype I VP3 we used the same approach as for generating pHB36-s2VP3N, only this time we used TY89 cDNA as template in the PCR and used pHB-36W as recipient for the 373 bp PCR fragment, yielding
15 pHB36-s2VP3C (see Table 9).

Protein analysis. For western blotting we purified CEF94 and TY89 IBDV from infected QM5 cells by differential
20 centrifugation as described before (Boot et al., 1999). Viral proteins were separated in a 12% SDS-PAGE gel, and blotted onto nitrocellulose (Protean BA85, Schleicher and Schuell). For detection of VP3 we used the ECL detection method (Amersham), using a VP3 specific monoclonal antibody, and peroxidase
25 conjugated rabbit anti mouse serum (Dako) as the second antibody.

For in vitro transcription and translation we used circular plasmids (0.4 ug) containing full-length A- or B-segments (see Fig. 7), in a 2.5 ml TnT-T7Quick reaction (Promega) in the
30 presence of ³⁵S-methionine. The resulting viral proteins were separated either directly or after immuno-precipitation in an SDS-PAGE gel (12%) and detected by autoradiography. For immuno-precipitation of in vitro produced proteins we used either polyclonal (PaVP3/4) or monoclonal antibodies directed against
35 VP3 of serotype I IBDV (Mab IV^{SI}) or serotype II (Mab VII^{SII}). RIPA buffer (Sambrook, Fritsch, and Maniatis, 1989) was used during all incubations and washing steps. Antigen-antibody complexes were purified from the mixture by addition of Protein

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A Sepharose CL-4B (Amersham Pharmacia, Sweden), and three washing steps were performed.

Infection and transfection of QM5 cells. QM5 cells were grown to 80% confluence in a 35 mm culture dish and infected with FPV-T7 (m.o.i. = 3). After one hour, the cells were washed twice with 3 ml of QT-35 medium and covered with 3 ml Optimem 1 (Gibco/BRL). In the mean time, DNA (1.0 ug) was mixed with 12.5 ul LipofectAMINE (Gibco/BRL) in 250 ul Optimem 1 and kept at room temperature for at least 30 min. The QM5 cells were subsequently covered with 2 ml of fresh Optimem 1 and the DNA/LipofectAMINE mixture was added. After overnight incubation (18h, at 37° C in 5.0% CO₂), the transfected monolayer was rinsed once with PBS, fresh QM5 medium (supplemented with 5% FCS and 2% ABII) was added, and the plates were further incubated at 37° C (5.0% CO₂). After 24h of incubation the plates were freeze/thawed once and the supernatant was filtered through a 200 nm filter (Acrodisc, GelmanSciences) to remove the Fowlpox virus (FPV-T7) and cellular debris. The cleared supernatant was either stored at -70° C or used directly for detection of rIBDV by inoculating a near confluent monolayer of QM5 cells with part of the cleared transfection supernatant. IBDV specific proteins were visualized after 24 or 48h in an immuno peroxidase monolayer assay (IPMA) using polyclonal VP3/4 or monoclonal VP3 specific antibodies as the first antibody, and horse radish peroxidase conjugated goat anti rabbit or anti mouse as the second antibody..

Maximum titers and single step growth curves. To determine maximum virus titers, we used the initially rescued virus to infect fresh QM5 cells. Cells were incubated in complete medium (37° C, 5.0% CO₂) for five days (or until complete CPE was visible), freeze-thawed once, and filtered through a 200 nm filter (Acrodisc). This procedure was repeated three times (3 serial passages). The amount of IBDV (TCID₅₀) after the final filtration was determined by infecting 96-well tissue culture plates, containing near confluent QM5 cells, with 10-fold

dilutions of the IBDV samples. The 96-well plates were incubated for 48 hours, and wells in which IBDV antigen was present were detected by an IPMA.

For comparison of the replication ability of mCEF94-s2VP3C with unmodified serotype I IBDV, we determined single step growth curves of rCEF94 and mCEF94-s2VP3C. QM5 cells (2×10^6) were grown overnight (16 h) in a 60 mm cell culture dish. The medium was subsequently removed and 1 ml PBS containing IBDV ($TCID_{50} = 7.0$) was used to cover the cells. After one hour the supernatant was removed, the cells were rinsed four times with 8 ml of PBS, and 5 ml complete medium was added. At different time points (1h, 5h, 10h, 15h, 25h, and 50h post infection) samples were taken from the supernatant and stored at -20°C . The viral titer in each sample ($TCID_{50}$) was determined in an IPMA (see above). Independent growth curve's were performed two (mCEF94-s2VP3C) or three (rCEF94) times, and standard deviations between the titers at each time point were determined.

20 Results

Cloning and analysis of the VP3 encoding region of a serotype II strain. To construct serotype I recombinant IBDV viruses containing the VP3 protein of serotype II we had to clone the corresponding cDNA of a serotype II strain. First we attempted to clone the full-length A- and B-segment cDNAs of TY89, the prototype serotype II IBDV strain (McFerran et al., 1980). To generate these cDNAs we followed the full-length RT-PCR protocol that we used to clone the A-segment of serotype I strains. Although we succeeded repeatedly in cloning the full-length B-segment cDNA of TY89 (data not shown) we were unable to obtain the full-length cDNA of the A-segment using this protocol. Therefore, we only amplified the VP3 encoding part of the TY89 A-segment. An RT-PCR fragment containing VP3 of TY89 was obtained three times independently and cloned in the pGEM-T Easy vector. The consensus sequence for this part of the TY89 genome was determined and the deduced amino acid sequence was compared with the corresponding sequence of CEF94, a cell culture adapted serotype I strain (Fig. 6). The cDNA encoding

the TY89 VP3 was subsequently used to generate mosaic A-segment cDNA plasmids which encodes either the complete VP3 of TY89 (pHB36-s2VP3) or the N-terminal part (101 amino acids, pHB36-s2VP3N) or the C-terminal part (155 amino acids, pHB36-s2VP3C) (Fig. 7).

After having verified that the obtained plasmids had the correct sequence they were used as templates in an in vitro transcription/translation reaction, followed by SDS-PAGE and autoradiography (Fig. 8a). The three proteins, pVP2, VP3 and VP4, which result from autocatalytic cleavage of the polyprotein, were present in all cases. The positions of the VP3 proteins derived from plasmids pHB36-s2VP3 and pHB36-s2VP3N were found at a higher position in the SDS-PAGE gel than VP3 of pHB-36W and pHB36-s2VP3C. A difference in migration during SDS-PAGE separation was also found between viral VP3 obtained from purified CEF94 and TY89 virus in a western blot analysis (Fig. 8b). This indicates that the observed difference in migration of VP3 from the mosaic full-length A-segment plasmids is not an experimental artifact, but due to amino acid differences in the N-terminal part of VP3

Three amino acid changes are present in the viral protease (VP4) of pHB-36W-s2VP3 and -s2VP3N (Fig. 6), which is due to the fact that the suggested cleavage site of the polyprotein (Hudson *et al.*, 1986) appeared not to be the genuine cleavage sites as recently has been shown (Sanchez and Rodriguez, 1999). These differences apparently do not alter the migration behavior of VP4 in an SDS-PAGE analysis, as this VP4 is found at the same position as wild-type serotype I VP4 (Fig. 8a). Also, no influence of these amino acid changes was found on the in vitro cleavage of the polyprotein, as no uncleaved or partially cleaved polyprotein was present (Fig. 8a).

Localization of a serotype specific epitope on VP3.

Immunodominant epitopes, recognized by non-neutralizing antibodies are present on VP3. At least one of these epitopes is serotype specific as has been reported by several researchers (Mahardika and Becht, 1995; Oppling, Muller, and Brecht, 1991; Reddy, Silim, and Ratcliffe, 1992). To map the

position of serotype specific epitopes and to assess their usefulness for discriminating virus rescued from mosaic A-segment cDNAs we performed a radio-immune-precipitation (RIP) of the in vitro translated and $\{^{35}\text{S}\}$ -Met labeled VP3 proteins, and used either a polyclonal (PaVP3/4) or monoclonal (Mab IV^{SI} or Mab VII^{SII}) antibody in the RIP, we analyzed the precipitated proteins by SDS-PAGE, followed by autoradiography (Fig. 8c). As expected from the high level of homology, VP3 derived from both serotype I and II was precipitated by polyclonal PaVP3/4 serum. However, monoclonal antibody IV^{SI} only precipitated VP3 that contained the C-terminal part of serotype I VP3, while Mab VII^{SII} precipitated solely VP3 that contained the C-terminal part of serotype II VP3. These results indicate that a serotype specific epitope is present in the C-terminal 155 amino acids of VP3.

Rescue of mosaic IBDV. The full-length mosaic A-segment cDNA plasmids were used to rescue mosaic IBDV (mCEF94) by co-transfecting these plasmids with a plasmid containing the B-segment cDNA of CEF94 (pHB-52, (Boot et al., 1999)) into QM5 cells. To direct viral RNA synthesis through the use of a T7-promoter, we infected the QM5 cells with a recombinant Fowlpox virus that carries the T7 polymerase gene (FPV-T7, moi = 3), prior to transfection. Expression of the polyprotein was examined in an IPMA 24h after transfection, and was found to be similar for all the used A-segment plasmids (about 30% of the cells expressed IBDV antigens, data not shown). The cleared supernatant of the transfected QM5 cells was used to determine the IBDV titer using fresh QM5 cells. Despite similar levels of expression of viral proteins after transfection, the resulting TCID₅₀ differed largely (Table 10). Recovery of mosaic virus having the C-terminal part of serotype II VP3 was as efficient as wild-type (Average TCID₅₀ = 4.8 compared to 5.1). However, the efficiency of recovering mosaic virus from plasmid pHB36-s2VP3 was strongly reduced (average TCID₅₀ = 1.1) compared to unmodified rCEF94 (average TCID₅₀ = 5.1). The same was found for the rescue of mosaic virus from pHB60-s2VP3N (average TCID₅₀ =

3.1), of which the titer was about 100-fold lower than unmodified rCEF94 after rescue (Table 10).

Replication of mosaic IBDV. To determine the effect of the

5 exchange of parts of the polyprotein on replication we determined the maximum titers of each virus after three serial passages. Although the mosaic virus which contained 3 amino acid changes in VP4, in combination with the N-terminal part of the serotype II VP3 did show an increase in viral titers after
10 three serial passages, the maximum TCID₅₀ values (6.0) obtained were about 10-fold lower than those of unmodified rCEF94 and of mosaic virus containing the C-terminal part of serotype II VP3 (Table 10). To investigate whether rCEF94 and mCEF94-s2VP3C, which were rescued with the same efficiency, had the same
15 replication kinetics in cell culture, we performed a single step growth curve with both viruses using QM5 cells. Although the maximum titers obtained were the same at 25h post infection (p.i.), there was a delay in virus release in case of mCEF94-s2VP3C in comparison unmodified rCEF94 (Fig. 9). The first
20 virus release after infection with unmodified rCEF94 was found at 10h p.i., while at that time point no release of virus was found after infection with mCEF94-s2VP3C.

25 **Antigen detection after infection with mosaic CEF94**

viruses. After rescue of the mosaic IBDV we assessed the possibility of discriminating between these IBDVs by using a set of VP3 specific monoclonal antibodies. Fresh QM5 cells were infected with the rescued viruses rCEF94, mCEF94-s2VP3, -
30 s2VP3N, and -s2VP3C, and viral antigens were detected at 48h p.i., by using IBDV specific monoclonal antibodies. Viral antigen originating from all the four different rescued viruses, was detected in infected QM5 cells when serotype I specific neutralizing VP2 antibodies (1.4 or 9.8, see Table 11)
35 or non-neutralizing non-serotype specific VP3 antibodies (9.7 or 17/80, see Table 11) were used. On the other hand, VP3 monoclonal antibodies which are serotype specific reacted differently with the four rescued viruses. Serotype I specific

antibodies (IV^{SI} or I/G4^{SI}, see Fig. 10) reacted only with rCEF94 and mCEF94-s2VP3N while the serotype II specific antibody (VII^{SI}, see Fig. 10) reacted only with mCEF94-s2VP3 and mCEF94-s2VP3C. The reaction pattern of the monoclonal antibodies used
5 was the same when tested in an RIP on in vitro synthesized IBDV proteins (Fig. 8c) and by an IPMA on infected QM5 monolayers (Fig. 6).

Vaccination with so-called deletion (or marker) vaccines
10 has been shown to be a very powerful tool for control and eradication of several viral animal diseases (van Oirschot, 1999). These vaccines are based on live (attenuated) virus strains which lack a non-essential, structural protein, allowing serologic discrimination of vaccinated and field virus
15 infected animals. Serologic discrimination between chickens vaccinated with a live IBDV vaccine strain and chickens infected with a wild-type IBDV field isolate is currently impossible, because all live IBDV vaccines are derived directly, or after serial passages from IBDV field isolates.
20 IBDV is a non-enveloped virus containing only three structural proteins (VP1, VP2 and VP3), which are all essential. Because it is not feasible to knock-out of one of the structural proteins of IBDV, we have used an alternative approach to introduce a serologic marker. Instead of deleting a viral
25 protein we have exchanged part of a structural protein by the corresponding part of a serotype II protein.

Several IBDV serotype specific properties, like neutralizing epitopes and receptor recognition, are located
30 within the hypervariable region of VP2. This difference in serotype specific properties of VP2 is reflected by the difference of the primary sequence of a hypervariable region present within VP2. The identity between the VP2 hypervariable region of the two serotypes is only 67%, while
35 the remaining parts of VP2 are identical for 93%. The other viral proteins also have an identity of over 90% (VP1 = 99%, VP3 = 95%, and VP4 = 92%), with the exception of VP5 which is only identical for 80%. Viral protein 3 is, just like VP2, a major constituent of the viral capsid. Based upon the fact

the VP3 has a basic C-terminal tail, which can interact with the packaged dsRNA, it has been proposed that VP3 constitutes the inner part of the virion (Bottcher et al., 1997). This hypothesis is further supported by the fact that 5 VP2 but not VP3 is involved in receptor recognition, which implicates that at least part of VP2 has to be present at the surface of the virion. Furthermore, neutralizing antibodies are elicited only by VP2 and not by VP3, which is also indicative for surface exposure of VP2. Given the 10 fact that no serotype specific properties are known for VP3, and the fact that at least one linear, serotype specific, non-neutralizing epitope, is present within this protein (see Introduction) makes the exchange of (part of) VP3 in our view the most promising approach to develop an IBDV 15 marker vaccine.

First, the VP3 encoding cDNA of TY89 was cloned by means of RT-PCR and its nucleotide sequence was determined (see Fig. 6 for the deduced TY89 amino acid sequence). This 20 cDNA was subsequently used to generate mosaic full-length A-segment cDNA plasmids (see Fig. 7). These full-length plasmids were analyzed for the production of viral proteins, in a combined in vitro transcription/translation reaction in the presence of [³⁵S]labeled methionine. Autocatalytic 25 cleavage of the polyprotein by VP4 was not affected by the (partial) exchange of VP3. Also the exchange of three amino acids in of serotype I VP4, just in front of the VP4-VP3 cleavage site of the polyprotein in plasmids pHB36-s2VP3 and -s2VP3N (see Fig. 6), did not influence the autocatalytic 30 cleavage. On the other hand, a quite remarkable difference in migration in SDS-PAGE between the in vitro synthesized VP3 originating from either serotype I or serotype II cDNA (Fig. 8a). The same difference in electrophoretic mobility was found for VP3 of purified TY89 and CEF94 (Fig. 8b), 35 although the predicted sizes of these proteins are similar (28.8 kDA). A similar difference in migration behavior between VP3 derived from serotype I and II IBDV has also been noted by others (Oppling, Muller, and Brecht, 1991;

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Reddy, Silim, and Ratcliffe, 1992). Furthermore, we observed two VP3 bands (VP3a and VP3b) in the Western blot analysis of purified CEF94 and TY89 (Fig. 8b). Whether these two VP3 bands both represent functional proteins, or whether the smaller band (VP3b) is just a specific degradation product of the larger band (VP3a) is unclear. We have observed the presence of two VP3 bands for cell culture amplified CEF94 previously Fahey et al. (Fahey, Erny, and Crooks, 1989) have made a similar observation for the Australian IBDV isolate 002/73, while also the presence of two VP3 bands for IPNV has been reported (Dobos, 1977) (Hjalmarsson and Everitt, 1999)

After in vitro characterization of viral proteins encoded by the full-length mosaic A-segments, we subsequently used these plasmids to rescue mosaic IBDV. The plasmid encoding the wild-type serotype I polyprotein (pHB-36W), and the three plasmids encoding a chimeric polyprotein (pHB36-s2VP3, -s2VP3N, and -s2VPC) were each co-transfected with the serotype I B-segment plasmid (pHB-52). We found that all transfections with full-length mosaic A-segment plasmids resulted in the appearance of infectious mIBDV. The rescue titers of mosaic viruses which carried the complete or the N-terminal part of serotype II VP3, in combination with three amino acid differences in VP4, were however considerably lower than those of unmodified rCEF94 or mCEF94 containing the C-terminal part of serotype II VP3. The difference in rescuing efficiency correlates with the maximum TCID₅₀ values obtained after 3 serial passages (Table 10). The introduction of the five amino acid differences in the N-terminal part VP3 alone or in combination with the three amino acid differences in VP4 leads apparently to a reduction in replication efficiency. Based upon the fact the polyprotein cleavage is not affected by the amino acid changes in VP4, and the fact serotype II VP3 behaves differently in an SDS-PAGE analysis, we think that the observed reduction in replication efficiency of mCEF94-s2VP3 and -s2VP3N is largely due to a different folding of the N-terminal part of VP3.

The first reports of a potential IBDV marker vaccine virus concerned a VP5 knock-out mutant of the attenuated, cell culture adapted rD78 vaccine strain (Mundt, Kollner, and Kretzschmar, 1997; Yao, Goodwin, and Vakharia, 1998).

5 Although a VP5-minus rIBDV might yield a good serological marker its efficacy as a vaccine has not been proven yet. Replication of VP5-minus rIBDV is severely affected both in vitro and in vivo. Mundt et al. (Mundt, Kollner, and Kretzschmar, 1997) reported that VP5-minus rD78 had a
10 highly reduced virus release (> 200-fold lower titer after 24h p.i. in a single step growth curve), while Yao et al. (Yao, Goodwin, and Vakharia, 1998) reported a large reduction in virus yield and a much higher survival rate of CEF cells (8-days p.i., multi-step growth curve),
15 in comparison to unmodified rD78. Although mCEF94-s2VP3C has a slightly retarded release of virus (Fig. 9), replication in cell culture is much less affected than that of the VP5 knock-out mutant. The finding that mCEF94-s2VP3C virus replicates almost as efficient as an
20 unmodified serotype I strain, in combination with the possibility to discriminate this virus from both serotype I and II wild-type field isolates, makes the use the C-terminal part of serotype II VP3 a very promising approach to develop an IBDV marker vaccine.

25

Example 2

**Exchange of the C-terminal part of VP3 results in an attenuated
very virulent IBDV vaccine strain that induces a unique
30 serologic response in chicken**

In this example we describe the replacement of the cDNA encoding the C-terminal part of VP3 of the very virulent IBDV isolate (D6948) with the corresponding part of serotype II
35 (TY89) cDNA. We were able to rescue the recombinant virus containing the serotype II C-terminal half of VP3 after transfection of QM5 cells and passage of the transfection supernatant either onto primary bursa cells or embryonated eggs. Infection of SPF-chickens with such a modified vvIBDV

(mD6948-s21VP3C3) shows a significant reduction in virulence compared to un-modified very virulent IBDV. As expected, we found that the distinct bursa tropism of serotype I IBDV strains was not affected by the introduction of the serotype II C-terminal part VP3. Oral administration of mD6948-s2VP3C3 to 18-days-old broiler-type chickens showed that this virus is able to infect chickens, despite the presence of low, intermediate, or high levels of maternally derived neutralizing IBDV antibodies.

Furthermore we show that the antibody response of chickens challenged with modified virus can be discriminated from the un-modified virus, using a competition Elisa test. The fact that the modified very virulent D6948 isolate has a slightly reduced virulence, combined with the ability to discriminate chickens infected with mD6948-s2VP3C3 from chickens infected with wild-type (very virulent) IBDV field viruses makes this mosaic IBDV isolate a valuable IBDV marker vaccine candidate.

20 Introduction

Infectious Bursal Disease Virus (IBDV) is the causative agent of a highly contagious disease among chickens known as Gumboro disease (Cosgrove, 1962). IBDV is a member of the family of *Birnaviridae*, having a double stranded RNA (dsRNA) genome divided over two segments (Dobos et al., 1979). The largest dsRNA segment (A-segment, about 3260 bp) contains two partly overlapping open reading frames (ORFs). The first, smallest ORF encodes the non-structural Viral Protein 5 (VP5, 145-149 amino acids, 17 kDa). The second ORF encodes a polyprotein (1012 amino acids, 110 kDa), which is autocatalytically cleaved to yield the viral proteins pVP2 (also known as VPX, 48 kDa), VP4 (29 kDa) and VP3 (33 kDa). During *in vivo* virus maturation pVP2 is processed into VP2 (41-38 kDa), probably resulting from site-specific cleavage of the pVP2 by a host cell encoded protease (Kibenge et al., 1997). The smaller B-segment (about 2827 bp) contains one large ORF, encoding VP1 (877-881 amino acids, 91 kDa). Viral protein 1 is, next to being the RNA dependent RNA polymerase, covalently linked to the 5'-ends of the genomic RNA

segments (Viral Protein genome-linked, VPg) (Dobos, 1993). *In vivo* expression of the polyprotein (VP2-VP4-VP3) already results in the formation of virus like particles, consisting of VP2 and VP3, which have the same dimensions as mature virions (60 nm).

- 5 This indicates that neither the free VP1, nor viral dsRNA is essential for the formation of the viral capsid (Fernandez-Arias *et al.*, 1998).

- Two different serotypes of IBDV have been described
- 10 (serotype I and II) (McFerran *et al.*, 1980). The pathogenic wild-type serotype I IBDV isolates have a specific tropism for lymphoid cells in the bursa of Fabricius. Serotype I isolates are sub-divided into classical, antigenic variant, and very virulent isolates. Antigenic variant IBDV isolates appeared to
- 15 have single amino acid changes in a specific region of the VP2 protein (the hypervariable region) leading to a partial change of proteins antigenicity (Snyder, 1990). Very virulent IBDV isolates, which were first isolated in Europe, have the same antigenic structure as classical strains, but have an enhanced
- 20 virulence (Chettle, Stuart, and Wyeth, 1989). Amino acid differences between viral proteins of vvIBDV and classical IBDV isolates are scattered throughout all viral proteins, although most of them are found in the hypervariable region of VP2 (Brown and Skinner, 1996). Specific mutations in VP2 result both in a
- 25 change of cell tropism (Mundt, 1999) and attenuation (Yamaguchi *et al.*, 1996c). Although VP2 is a key factor for virulence, we showed recently that VP2 is not the sole determinant for the very virulent phenotype.

- 30 Unlike serotype I isolates, wild-type serotype II isolates do not have a specific B-lymphoid cell tropism. Serotype II isolates are naturally able to replicate in different tissues of the birds and can even be propagated on cell lines. Serotype II isolates are usually recovered from turkey and are neither
- 35 pathogenic for turkey (Jackwood *et al.*, 1984) nor chickens (Ismail, Saif, and Moorhead, 1988). Conformation dependent, virus-neutralizing epitopes of both serotype I and II isolates

are present on the capsid protein VP2. The other abundant viral capsid protein (VP3) does not contain virus-neutralizing epitopes, although a fast immune response is found against linear VP3 epitopes both after vaccination and infection (Fahey, 5 IJ, and Bagust, 1985). Both serotype specific and non-serotype specific epitopes have been described for VP3 (Mahardika and Becht, 1995; Oppling, Muller, and Brecht, 1991; Yamaguchi et al., 1996a).

10 A common way of producing IBDV vaccines is the adaptation of wild-type virus by propagation in chicken embryos or in cell culture using primary chicken embryo cells or cell lines of either avian or mammalian origin. Adaptation of wild-type IBDV correlates always with a reduced virulence (attenuation)
15 (Yamaguchi et al., 1996b; Yehuda et al., 1999). Differentiation between the used live IBDV vaccines and field IBDV isolates recovered during field outbreaks is currently difficult and laborious as the genetic structure of the IBDV isolate in question has to be determined. In search for an IBDV vaccine
20 which can easily be distinguished from IBDV field isolates, we recently have replaced the complete or the N- or C-terminal half of the VP3 protein of an attenuated IBDV (CEF94) isolate by the corresponding part of a serotype II (TY89) isolate. Analysis of the rescued recombinant serotype I IBDV showed that virus
25 containing the C-terminal part of serotype II VP3 has a slightly reduced virus release when propagated in cell culture, while the final titer is equal to un-modified serotype I IBDV. The mosaic virus in which either the complete or the N-terminal half of VP3 was replaced has a reduced yield (final titers are 10-fold
30 lower) compared to un-modified CEF94, indicating a more severe interference in replication.

Material and Method

35 **Viruses, cells, and antibodies.** The classical IBDV isolate CEF94 is a derivative of PV1 (Italy, 1973 (Petek, D'Aprile, and Cancellotti, 1973)), which is able to replicate on non-B-lymphoid cells (Boot et al., 1999). The D6948 strain is a

very virulent field isolate (Poultry Health Service, The Netherlands, 1989), which is only able to grow on B-lymphoid cells. Recombinant Fowlpox virus containing the T7 polymerase gene (FP-T7) (Britton et al., 1996) was a kind gift of M.

- 5 Skinner (Compton Laboratory, Berks, United Kingdom). QM5 cells (a quail cell line (Antin and Ordahl, 1991) were maintained by using QT35 medium (Gibco-BRL), supplemented with 5% Fetal Calf Serum (FCS) and 2% antibiotic solution (QT35^{FA} medium). Primary bursa cells were isolated from 14-days old SPF embryos and were
- 10 maintained in Eagle's modified Minimal Essential Medium (EMEM) supplemented with 15% FCS, 0.125% Lacto Albumine Hydrolysate, and 1000 Units Penicillin and 1 mg Streptomycin per ml. The VP3 specific monoclonal antibodies B10A, IV, VII (Mahardika and Becht, 1995), and I/G4 (Oppling, Muller, and Brecht, 1991) were a
- 15 kind gift of Dr. Müller, University of Leipzig (Germany), while VP3 monoclonal antibody 9.7 was prepared in our laboratory.

- Construction of chimeric VP3 cDNAs.** The construction of hybrid CEF94 and TY89 plasmids have been described before. For
- 20 the construction of plasmid pHB60-s2VP3N we generated 5 different PCR fragments. In all PCR's we have used Pwo polymerase during 20 reaction cycles. PCR-1N was made using pHB-60 as template and primers AC3 (GGTAGCCACA TGTGACAG) and HY3MR (CCAGTCCCCGC GGATTGTGAGG) at 56°C, yielding a 1614 bp fragment (nt
- 25 731-2346). PCR-2N was made using pHB36-s2VPN as template and primers HY3P (AACGTTTTC TCACAATCCG CGGGACTGGG) and M13F-17 (GTAAAACGAC GGCCAGT) at 56°C, yielding a 1251 bp fragment (nt 2318-3566). PCR-3N was made using gel purified PCR-1N and PCR-2N fragments as template and primers AC4 (ACCCAGCCAA TCACATCC) and
- 30 AGTM (GAGACTCCCA GGTACCTCAC TC) at 54°C, yielding a 2151 bp fragment (nt 1057-3208), which was subsequently digested with ScaI (nt 2799), yielding PCR-3sN. PCR-4N was made using pHB-60 as template and primers AC9 (CTCAAAGAAG ATGGAGACC) and M13F-24 (CGCCAGGGTT TTCCAGTCA CGAC) at 54°C, yielding a 869 bp fragment
- 35 (nt 2727-3593). PCR-5 was made using gel purified PCR-3sN and PCR-4 fragments as template and primers AC5 (AAGGCCTTCA TGGAGGTGGC CG) and M13F-17 (GTAAAACGAC GGCCAGT) at 54°C, yielding a 2143 bp fragment (nt 1423-3566), which was subsequently

digested with *Xho*I and *Xba*I, yielding a 1889 bp PCR-5xxN fragment (nt 1606-3495). The PCR-5xxN fragment was subsequently used to replace the corresponding part of pHB-60 using the rapid DNA ligation kit and transformation of *E. coli* cells. The DNA
5 sequence of the selected plasmid clone of pHB60-s2VP3N was determined (nt 1600-3325), and appeared to have the intended mosaic D6948-TY89 cDNA sequence as shown in Fig.1.

For the construction of pHB60-s2VP3C3 we first transferred a 1735 bp fragment of pHB36-s2VP3C into pHB-60 using the unique
10 *Sac*II (1670) and *Xba*I (3495) sites, yielding pHB60-s2VP3C1. The 3'UTR region of CEF94 was subsequently replaced by the corresponding cDNA sequence of D6948. A PCR fragment (385 bp, nt 3184 -3566) was generated by using primers AGTP (CTTGAGTGGAG GTACCTGGGAG) and M13F-17 (GTAAAACGAC GGCCAGT), using pHB-60 as
15 template, with a hybridization temperature of 52° C, *Pwo* polymerase, and 20 reaction cycles. This PCR fragment was purified, and digested with *Kpn*I and *Xba*I. The resulting fragment (315 bp, nt 3198-3519) was gel purified, and used to replace the corresponding part of pHB60-s2VP3C1 (digested with
20 *Kpn*I and *Xba*I), by using rapid ligation and transformation of *E. coli* cells, yielding pHB60-s2VP3C2. Next, a PCR fragment (1187 bp, nt 1423-2607) was generated by using primers AC5 (AAGGCCTTCA TGGAGGTGGC CG) and vvVP3CM (GAGAAAATTT CGCATCCGATG) using pHB-60 as template, with a hybridization temperature of 54° C, *Pwo*
25 polymerase, and 20 reaction cycles. This PCR fragment was purified (High Pure PCR purification, Boehringer Mannheim), digested with *Sac*II (nt 1760) and *Apo*I (nt 2576). The resulting 816 bp fragment was used to replace the corresponding part of pHB60-s2VP3C2 (digested with *Apo*I (nt 2576, partially) and *Sac*II
30 (nt 1760)), by using rapid ligation and transformation of *E. coli* cells, yielding pHB60-s2VP3C3. The DNA sequence of the selected plasmid clone of pHB60-s2VP3C3 was determined (nt 1600-3275), and appeared to have the intended mosaic D6948-TY89 cDNA sequence as shown in Fig.1.

35

Infection and transfection of QM5 cells. QM5 cells were grown to 80% confluency in a 35 mm culture dish (M-6, 9.6 cm²/well) and infected with either Fowlpox virus-T7 (FP-T7)

- (Britton et al., 1996) or Modified Vaccinia Ankara (MVA-T7) (Wyatt, Moss, and Rozenblatt, 1995) with a multiplicity of infection (m.o.i.) of 3. After one hour, the cells were washed twice with 3 ml of QT-35 medium and covered with 3 ml Optimem 1 (Gibco/BRL). In the mean time, DNA (1.0 mg) was mixed with 12.5 ml LipofectAMINE (Gibco/BRL) in 250 ml Optimem 1 and kept at room temperature for at least 30 min. The QM5 cells were subsequently covered with 2 ml of fresh Optimem 1 and the DNA/LipofectAMINE mixture was added. The transfection was performed overnight (18h) in a 37° C incubator (5.0% CO₂). The transfected monolayer was rinsed once with PBS, and fresh QM5^{FA} medium (2.5 ml) was added and the plates were further incubated in the 37° C (5.0% CO₂) incubator. After 24h of incubation the plates were freeze/thawed once and the supernatant was filtered through a 200 nm pore-size filter (Acrodisc, GelmanSciences) to remove fowlpox virus and cellular debris. The cleared supernatant was either stored at -70° C or used directly for further analysis.
- Detection of infectious rIBDV.** After transfection, cell culture adapted rIBDV was detected by inoculating a near confluent monolayer of QM5 cells with 10-fold dilutions of the cleared transfection supernatant. IBDV specific proteins were visualized after 24h in an Immuno-Peroxidase Monolayer Assay (IPMA) (Boot et al., 1999). Rescued IBDV (e.g. rD6948), which is unable to replicate on QM5 cells was used to inoculate a monolayer of primary bursa cells which had been grown in vitro for 24h in a 35 mm tissue culture dish (10 cm²) in a CO₂ incubator (5%) at 39° C. IBDV specific proteins in infected B-lymphoid cells were detected in an IPMA after 48h using Mab 9.7, which is a non-serotype specific VP3 antibody.

- Virulence of rIBDV in young SPF chickens.** The virulence of the rescued IBDV isolates was evaluated in SPF layer-type chickens. The IBDV isolates were first propagated on embryonated eggs, by inoculating the QM5 cell supernatant of a transfection experiment into 11-days old embryonated eggs via the

chorioallantoic membrane route. After five days of incubation the embryos (dead or alive) were recovered, homogenized in a Sorval Omni-mixer (3 * 10 sec, max. speed) and subsequently clarified by centrifugation (6000 g, 10 min.), and stored in aliquots at -70° C. The virus titers (50% embryo lethal dose, ELD₅₀) in these samples were determined using 11-days old embryonated eggs. The groups of (21-days old) chickens were housed separately in isolators and inoculated intranasally and by eye-drop with 1000 ELD₅₀ IBDV in PBS, or only PBS. (negative control group). The animals were monitored for clinical signs daily and dead chicks were removed and necropsied. In experiment 1 most surviving birds were bled (5 ml) and euthanized for necropsy at 9 days post infection (p.i.), while some birds were left until day 15 p.i. to be bled and euthanized (see Table 13). In experiment 2 all surviving birds were bled and euthanized for necropsy at 13 days (p.i.) infection. Bursa and body weights were determined of all chicks euthanized, and viral neutralizing antibody titers in sera were determined using 100 TCID₅₀ of CEF94 and a confluent monolayer of QM5 cells. Samples from the bursa of Fabricius taken at necropsy were fixed in 10% neutral-buffered formalin for histopathology, and samples were also snap frozen in liquid nitrogen and preserved at -70° C for immunohistochemistry (only Exp. 1).

Histopathology and immunohistochemistry. Formalin fixed bursa samples were dehydrated, embedded in paraffin wax, sectioned and stained with Hematoxylin-Eosin (H & E). Histopathologic bursal lesion score (HBLS) was determined by microscopic analysis of the bursa. HBLS was determined on a scale of 1 to 5 according to (Bayyari et al., 1996): 1 = normal bursa; 2 = scattered or partial follicle damage; 3 = 50% or less follicle damage; 4 = 50-75% follicle damage; 5 = 75-100% follicle damage. Frozen bursa samples were sectioned for immunohistology on a cryostat at 8 µm thickness and taken up on glass slides (Superfrost®). The sections were fixed with acetone for 10 min, air-dried and stored at -20 °C until used. An immunoperoxidase staining was performed as described (Pol, Gielkens, and Van Oirschot, 1989). Briefly, endogenous peroxidase in sections was

eliminated by 0.01% H₂O₂. Non-specific reactions were blocked by 0.2% bovine serum albumin. Mab B10A or IV^{SI} was used as the primary antibody, whereas goat-anti-rabbit or rabbit-anti-mouse antibody conjugated to peroxidase (Dako) was used as the secondary antibody, and Diamino-benzidine (DAB) as substrate.

Infection of young broilers which posses maternally derived IBDV antibodies. Serum of young broilers derived from breeders, which had been vaccinated with both live and killed IBDV vaccines, were assessed for the presence of maternally derived antibodies at 7 and 15 days post hatch (p.h.). The level of IBDV specific antibodies were determined both by using the IBDV specific Elisa of Idexx (Idexx, Westbrook, USA) and by using a virus neutralization test. In the virus neutralization test we used 100 TCID₅₀ of CEF94 and a confluent monolayer of QM5 cells.. Based upon the results of the IBDV Idexx Elisa at 15 days p.h. we grouped the animals in five different groups of at least 14 animals (see Table 14) and housed the groups separately in isolators. At 18 days p.h. each individual chicken received 1000 ELD₅₀ of mD6948-s2VP3C3 orally in the crop. Blood samples were taken of each chicken at 22, 25, 32, and 42 days p.h. and the levels of IBDV antibodies was determined in each sample in the IBDV Idexx Elisa and in the virus neutralization test. Five birds of each group were euthanized after the blood sample of 22 days p.h., while the remaining birds were euthanized after the blood sample of 42 days p.h. The bursa weight body weight ratio was determined for each bird which was euthanized at 41 days p.h.

Results

Construction and analysis of chimeric A-segment plasmids.

To assess the viability, virulence and antigenic properties of IBD viruses containing a chimeric A-segment dsRNA, we have made several plasmids which contain a full length A-segment cDNA encoding a hybrid polyprotein. Next to A-segment plasmids based upon CEF94 cDNA, and encoding a VP3 which is (partially) derived from the TY89 serotype II isolate, we constructed three chimeric

plasmids which are based upon A-segment plasmid of D6948, a very virulent IBDV isolate. Two of these plasmids (pHB60-s2VP3C1 - s2VP3C3, see Fig. 11 and Table 12) encoded a polyprotein in which the C-terminal part of the VP3 is derived from serotype II, while the other (pHB60-s2VP3N, Fig. 11 and Table 12) encodes a polyprotein in which the N-terminal part of VP3 originates from serotype II IBDV. All plasmids encoding the hybrid VP3 proteins possess a T7 promoter and the hepatitis delta virus ribozyme. T7 RNA polymerase driven transcription of these plasmids leads to positive stranded A-segment IBDV RNA which exactly mimics the nucleotide sequence of the viral positive stranded RNA. A difference is however present between native viral RNA and artificially produced viral-like RNA, because a viral protein genome linked molecule (VP1), is present at the 5'-end of native RNA which lacks in the artificial produced RNA.

The constructed plasmids were subsequently used to transfect QM5 cells, which were infected prior to transfection with a recombinant poxvirus (either fowlpox (FP-T7 or vaccinia MVA-T7) which expresses T7 polymerase cytoplasmatically after infection. No replication of the resulting A-segment RNA can occur as the RNA dependent RNA polymerase (VP1), encoded by the B-segment, is absent. After transfection, the cells were washed and the total cell extract was assessed for the presence of the (recombinant) VP3 by Western blot analysis (Fig. 12). Four different monoclonal antibodies, known to react (serotype specific) with VP3 were used. Monoclonal B10A is a non-serotype specific monoclonal antibody, and reacted with VP3 encoded by all the used plasmids. The serotype I specific monoclonal IV^{SI} reacts only with those VP3's which possess the C-terminal part of serotype I, while monoclonal I/G4^{SI} only recognizes the VP3 C-terminal part of the cell culture adapted classical serotype I isolate (CEF94), and not with the very virulent serotype I (D6948) derived VP3. The recognition pattern of the serotype II specific monoclonal T75^{SI} is just the opposite of monoclonal IV^{SI}, it recognizes exclusively those VP3's which possess the serotype II C-terminal part.

Amino acid sequence comparison. Based upon the Western blot analysis we concluded that the VP3 epitopes, which are recognized by the three serotype specific Mabs, are linear and present in the C-terminal part (amino acid 850-1012) of this protein. An alignment of the deduced amino acid sequences of the VP3 part of the polyprotein reveals that only two amino acids are different between the two serotype I isolates CEF94 and D6948 (i.e. amino acid 981 and 1005; see Fig. 13). The fact that monoclonal I/G4^{SI} only recognizes CEF94 and not D6948 is apparently due to one of these amino acid differences. Furthermore, I/G4^{SI} is also unable to react with TY89. Several differences are present between the VP3 of the serotype II and serotype I strains. Because the epitope for I/G4^{SI} is present either around amino acid 981 or 1005 (see above), we concluded that the amino acid difference at position 981 or 992 (see Fig. 13) is responsible for the difference in reactivity of I/G4^{SI}.

Rescue of infectious mosaic IBDV. The full-length hybrid A-segment plasmids were subsequently transfected into FP-T7 or MVA-T7 infected QM5 cells, in combination with a full length B-segment plasmid (either pHB-34Z or pHB-55). Infectious mosaic IBDV could be rescued 24h after transfection, either using fresh QM5 (for all mCEF94 derivatives) or primary bursa cells (for mD6948-s2VP3C1 and -s2VP3C3) (Table 12). No virus could be rescued when plasmid pHB60-S2VP3N was transfected in combination with plasmid pHB-55. This either means that this mosaic virus is not viable, or that our method to rescue this virus is not efficient enough. The rescued viruses and the parental wild-type strains were all amplified on embryonated SPF eggs and harvested. The viral titer (50% embryo lethal dose (ELD₅₀)) for each virus stock was subsequently determined using embryonated SPF eggs.

Infection of SPF chickens with mosaic IBDV. To assess the virulence of rescued mosaic IBDV strains we inoculated (internasally and intraocularly) groups of SPF chickens with 1000 ELD₅₀ of wild-type or mosaic virus strains and determined several relevant parameters both during the living phase, or

post-mortem (Table 13). In the first animal experiment (Exp. 1) we observed mortality only in groups which received D6948 (either wild-type or rescued) during the first five days after inoculation. No mortality occurred in this experiment when the D6948 derivative mD6948-s2VP3C1 was used to inoculate the chickens. This is in contrast to the second animal experiment (Exp. 2) in which one chicken (out of 15) of this group died within 5 days p.i.. In Exp. 2 we found no mortality after infection with mD6948-S2VP3C3, a derivative of D6849 which is more close to the wild-type D6948 than mD6948-s2VP3C1 (see Fig. 11 and Table 13). The bursa and body weights of each chicken were determined after euthanization (Table 13). The group average of the ratio between the bursa weight and body weight is a good indication for the damage of the bursa of the SPF chickens, due to the IBDV infection. Cell culture adapted IBDV strains (e.g. CEF94), which do no longer possess a specific tropism for developing B-lymphoid cells in the bursa of Fabricius, induced only a minor reduction of this ratio (Table 13), while non-cell culture adapted IBDV strains (e.g. the very virulent D6948, and derivatives thereof), which possess the specific bursa tropism, induced a large reduction of the bursa weight body weight ratio. The introduction of the C-terminal part encoding part of the serotype II VP3 into the genome of vvIBDV (mD6948-s2VP3C3) did not alter this ratio, consistent with the fact that tropism for the bursa correlates with the sequence of VP2. Despite the fact that the very virulent VP2 sequence is present in mD6948-s2VP3C1, a difference is found for the bursa weight body weight ratio of mD6948-s2VP3C1 in comparison to wild-type or rescued D6948. This specific combination of the D6948, CEF94 and TY89 genomes apparently influences viral replication and hence bursa damage in this animal experiment (Exp. 2).

Immunohistochemistry of infected bursa of Fabricius.

Sections of the bursa of Fabricius of IBDV infected chickens (rD6948 or mD6948-s2VP3C1, Exp. 1) were used to show the presence of viral antigen after infection. Monoclonal antibodies either non-serotype specific (B10A) or serotype I specific (IV^{SI}) were used to show the distribution of viral antigen throughout

the infected bursa. Using Mab B10A, we were able to detect viral antigen in the bursas of both rD6948 and mD6948-s2VP3C3 infected chicken. When Mab IV⁸¹ was used no viral antigen could be detected in when bursas from mD6948-s2VP3C3 infected chicken, while viral antigen was clearly detected in bursas of rD6948 infected chickens (data not shown).

Histopathology of infected bursa. Sections of the bursa of Fabricius of IBDV infected chickens (Exp. 2) were examined after euthanization to determine the severity of bursa damage (Histopathological Bursa Lesions Score, HBLS). Bursa were classified according to Bayyari (Bayyari et al., 1996) from 1 for a normal bursa to 5 for 75-100% follicle damage (see Material and Methods). All viruses having the VP2 of CEF94 induced only minor bursa damage (< 2.0), while all viruses having a VP2 of D6948 induced severe bursa damage (4.7 - 5.0) (Table 13). Based upon the HBLS it seems that bursa damage is almost identical after mD6948-s2VP3C1 and -s2VP3C3 infection, in comparison to unmodified D6948. However, careful examination reveals some clear differences between the induced damage of the bursa follicles by the different D6948 based viruses (Fig. 14). Both D6948 and rD6948 completely destroyed the follicular structure in the bursa and induced necrosis and cystic formation. The overall follicular structure of the bursas of mD6948-s2VP3C1 and -s2VP3C3 infected chickens is however still present. Some follicles of the mD6948-s2VP3C1 infected bursa have the same appearance as found in control bursas (PBS group), although the diameter of these follicles is generally reduced in comparison to wild-type follicles (see Fig. 14). We also found that the length of the bursa villi is reduced in (m)D6948 infected chickens, in comparison to mock infected chickens. The reduction in the length of the villi in (m)D6948 infected bursas correlates with the reduced weight (and volume) of these bursas.

Infection of young broilers which possess maternally derived IBDV antibodies. After having verified that mD6948-s2VP3C3 has a reduced virulence (attenuation) in comparison to wild-type D6948, we assessed whether this virus is able to infect and

induce IBDV neutralizing antibodies in young chickens which possess maternal derived IBDV antibodies (animal Exp. 3). Five groups of at least 14 young chickens were formed on the basis of the IBDV specific antibody levels (IBDV Idexx Elisa) in the bloodserum samples taken 15 days post hatch (p.h.) (see Table 14). At day 18 p.i. each chicken was infected with mD6948-s2VP3C3 (1000 ELD₅₀) and the level of IBDV specific antibodies was determined both by Elisa (Idexx, see Table 14) and by virus neutralization (see Table 15. The level of IBDV antibodies as determined by the Idexx Elisa correlated overall quite well with the level of virus neutralizing antibodies as determined with the virus neutralizing assay (Table 14 and 15). When the ²log virus neutralizing antibodies titers are below 8.0, the Idexx Elisa gives a negative score (taken as 0).

In this animal experiment (Exp. 3) we found that mD6948-s2VP3C3 is able to infect birds which possess no (group 1, average Idexx value at 3 days pre-infection is 18), intermediate (group 2, average IBDV Idexx at 3 days pre-infection is 361) or high (group 5, average IBDV Idexx value at 3 days pre-infection is 1283) average level of maternally derived antibodies (Table 14). That the chickens in groups 1, 2 and 5 had indeed been infected is also clear from the average of the calculated bursa weight body weight ratio (BBR) of the chickens euthanized at 42 days p.h.. For groups 1 (BBR=0.57; SD=0.21), 2 (BBR=0.69; SD=0.25) and 5 (BBR=0.70; SD=0.16) these values are reduced compared to the non infected groups 3 (BBR= 2.22; SD=0.42) and 4 (BBR=2.17; SD=0.68). Although mD6948-s2VP3C3 was apparently able to infect the birds of group 5, which had the highest average level of maternal derived antibodies, no infection had taken place in groups 3 and 4, which have a intermediate to high average level of maternal derived antibodies (average IBDV Idexx value at 3 days pre-infection is 576 and 899 for groups 3 and 4, respectively). Apparently there is no strict correlation between the levels of maternal derived (neutralizing) antibodies and the ability to of mD6948-s2VP3C3 to infect the chick.

Outbreaks of very virulent IBDV (vvIBDV) among commercial chicken flocks were first reported in Europe, but are now found world-wide. Very virulent IBDV strains are able to infect young

- chicks despite a relatively high titer of maternally derived IBDV antibodies (MDIA) which are protective for infection with classical IBDV isolates. To prevent vvIBDV outbreaks, vaccines based upon wild-type (vv)IBDV isolates (hot vaccines) are being administered when the young chicks still have high levels of MDIA. In flocks with a high variation coefficient of MDIA, vaccination with a hot vaccine may lead to severe bursa damage in chicks with low levels of MDIA.
- Furthermore, the introduction of live, wild-type vvIBDV as vaccine into the field is unwanted by the animal health authorities, as transmission can not be prevented and no discriminating test are available to distinguish between vaccine and field virus.
- In this example we described the rescue of very virulent derived mosaic IBDV (mD6948-s2VP3C3), which has a VP3 that is based partly (the C-terminal 155 amino acids) upon the VP3 sequence of the serotype II TY89 strain. Earlier we described the generation and in vitro analysis of a mosaic virus (mCEF94-s2VP3C), based upon the classical attenuated serotype I strain CEF94, which has the same C-terminal exchange of the VP3 coding sequence (the C-terminal 155 amino acids) as mD6948-s2VP3C3. Replication of this virus appeared to be slightly reduced, as no virus release was found at 10 h post infection of a monolayer of QM5 cells. This is in contrast to wild-type CEF94, which showed a virus release at 10 h p.i.. Equal final IBDV titers ($> 10^7$) were reached both for wild-type CEF94 and mCEF94-s2VP3C between 25 and 50 h p.i., indicating that replication of mCEF94-s2VP3C is only minor effected by the exchange of the C-terminal 155 amino acids of VP3 for those of TY89. Here we show that mD6948-s2VP3C3 has a reduced virulence in comparison to wild-type D6948. This conclusion is based upon the fact that i) it does not induce any mortality when administered to 21-old layer-type SPF chicks, while wild-wild type D6948 induced mortality in about 50% of the birds (Table 13), ii) The induced bursa damage after infection of 21-old days SPF-chickens with mD6948-s2VP3C3 is reduced in comparison to infection with D6948 (Fig. 14). The introduction of the C-terminal part of VP3 serotype II in the

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back-ground of a serotype I isolate leads apparently both in vivo and in vitro small but distinct phenotype.

In this example we furthermore show that mD6948-s2VP3C3 is able to infect young chicks which possess maternally derived IBDV specific neutralizing antibodies. This particular mosaic virus infected all broiler-type non-SPF chicks in three groups which possessed either an average Idexx IBDV antibody titer of 18 (group 1, Table 14), 361 (group 2, Table 14) or 1283 (group 5, Table 14), at 3-days pre-infection. A distinct raise in the IBDV specific antibody levels is present between 4 and 7 days post infection in some of the animals belonging to the group 1 and 2, while all animals of these groups have high IBDV antibody levels between 7 and 14 days post infection. The IBDV specific antibody titer was found to raise between 14 and 24 days post infection in group 5, indicating that infection proceeded at a slower rate in this group than in groups 1 and 2. The average IBDV Idexx value of group 5 is far higher (1283 at 3 days pre-infection) then found for groups 1 (18 at 3 days pre-infection) and 2 (361 at 3 days pre-infection). The difference in level of the maternal derived antibody titers results most likely in the observed delayed in the raise of active (neutralizing) IBDV antibodies levels in group 5 in comparison with groups 1 and 2.

Sera of animals of experiment 2 were used in a competition Elisa, in which we used prokaryotically expressed C-terminal 290 amino acids of the CEF94 polyprotein as the coated antigen. Sera chickens infected with D6948 and mD6948-s2VP3C1 were used to compete with either monoclonal antibodies IV or I/G4. At a dilution of 1:500 of the chicken sera we found that sera from chickens infected with mD6948-s2VP3C1 were not able to compete with of Mab IV and I/G4 in the interaction with the coated antigen, while sera of D6948 infected chicken were able to compete with this interacting (Fig. 15). This indicates that the introduction of the serotype II sequences into the cDNA of D6948 leads to a mosaic virus which induces a unique antibody response. Using the described competition Elisa we are able to distinguish

between chickens infected with the wild-type serotype I vvIBDV virus (strain D6948) and the mosaic serotype I-II, attenuated vvIBDV virus (strain mD6948-s2VP3C1). Despite the fact the I/G4 did not react in the Western blot analysis with VP3 derived from pHB60 (Fig. 12), we found that antibodies induced by the derived virus of pHB60 (rD6948) were able to compete for the CEF94 antigen. The fact that the mosaic very virulent D6948 isolate (mD6948-s2VP3C3) has a reduced virulence (attenuated), combined with the ability of this virus to infect young chicks which have low, intermediate, or high levels of maternally derived IBDV neutralizing antibodies makes this virus a prime candidate for an efficacious vaccine to prevent outbreaks of vvIBDV. The ability to discriminate between chickens vaccinated (infected) with this virus from chickens infected with wild-type (very virulent) viruses makes this mosaic (vv)IBDV virus even more valuable, as it can be used to detect and follow (sub)-clinical (vv)IBDV infections in vaccinated flocks using our described competition Elisa. This (vv)IBDV marker vaccine also enables Gumboro eradication programs.

Example 3

Many recent outbreaks of infectious bursal disease in commercial chicken flocks world-wide are due to the spread of very virulent strains of infectious bursal disease virus (vvIBDV). The molecular determinants for the enhanced virulence of vvIBDV compared to classical IBDV are unknown. The lack of a reverse genetics system to rescue vvIBDV from its cloned cDNA hampers the identification and study of these determinants. In this report we describe, for the first time, the rescue of vvIBDV from its cloned cDNA. Two plasmids containing a T7 promotor and either the full-length A- or B-segment cDNA of vvIBDV (D6948), were co-transfected into QM5 cells expressing T7 polymerase. The presence of vvIBDV could be detected after passage of the transfection supernatant to either primary bursa cells (*in vitro*) or embryonated eggs (*in vivo*), but not QM5

cells. Rescued vvIBDV (rD6948) appeared to have the same virulence as the parental isolate D6948. Segment reassorted IBDV, in which one of the two genomic segments originated from cDNA of classical attenuated IBDV CEF94 and the other from D6948 could also be rescued by using this system. Segment reassorted virus containing the A-segment of the classical attenuated isolate (CEF94) and the B-segment of the very virulent isolate (D6948) is only released after 15h after an *in vitro* infection. This indicates a slightly retarded replication, as the first release of CEF94 is already found at 10h after infection. Next to segment reassortants, we generated and analyzed mosaic IBDV. In these mosaic IBDV's we replaced the region of CEF94 encoding one of the viral proteins (pVP2, VP3, or VP4) by the corresponding region of D6948. Analysis of these mIBDV isolates showed that tropism for non-B-lymphoid cells was exclusively determined by the viral capsid protein VP2. However, the very virulent phenotype was not solely determined by this protein, since mosaic virus containing VP2 of vvIBDV neither induced morbidity nor mortality in young chickens.

Introduction

Infectious Bursal Disease Virus (IBDV) is the causative agent of a highly contagious disease among chickens known as Gumboro disease (11). IBDV is a member of the family of *Birnaviridae*, having a double stranded RNA (dsRNA) genome divided over two segments (14). The dsRNA genome is covered by a capsid of two viral proteins, which results in a single-shelled naked virus particle (60 nm) with an icosahedral (T=13) symmetry (5). The largest dsRNA segment (A-segment, about 3260 bp) contains two partly overlapping open reading frames (ORFs). The first, smallest ORF encodes the non-structural Viral Protein 5 (VP5, +/- 145 amino acids, 17 kDa, see Fig. 16a). The second ORF encodes a polyprotein (1012 amino acids, 110 kDa, see Fig. 1b), which is autocatalytic cleaved to yield the viral proteins pVP2 (also known as VPX, 48 kDa), VP4 (29 kDa) and VP3 (33 kDa) VP2, VP3, and VP4. During *in vivo* virus maturation

pVP2 is processed into VP2 (41-38 kDa), probably resulting from site-specific cleavage of the pVP2 by a host cell encoded protease (19). VP2 and VP3 are the two proteins that constitute the shell of the virion. Neutralizing antibodies are only known for VP2, and these antibodies are conformation dependent. The B-segment (about 2827 bp) contains one large ORF, encoding the 91 kDa VP1 protein (see Fig. 16c). This protein contains a consensus RNA dependent RNA polymerase motif (8). Furthermore, this protein has been reported to be linked to the 5'-ends of the genomic RNA segments (Viral Protein genome-linked, VPg) (12, 29).

The pathogenic serotype I IBDV isolates are sub-divided into classical, antigenic variant, and very virulent isolates. Antigenic variant IBDV's have only been reported in the USA (since 1985) and were found to have single amino acid changes in a specific region of the VP2 protein (the hypervariable region), leading to a different pathologic phenotype (28). Later on in Europe (since 1988 (9)), reports appeared describing IBDV isolates that had an enhanced virulence (very virulent IBDV, vvIBDV), while having the same antigenic structure as classical isolates. Amino acid differences between viral proteins of vvIBDV and classical IBDV isolates were found scattered throughout all viral proteins, although most of them were found in the hypervariable region of VP2 (7, 24). It is currently unknown whether all, or only a few of these amino acid mutations contribute to the enhanced virulence of the vvIBDV isolates.

Wild-type IBDV replicates specifically in developing B-lymphoid cells in the bursa of Fabricius. During this replication, viral proteins induce apoptosis resulting in a rapid depletion of B-cells (31). A common way of producing IBDV vaccines is the adaptation of wild-type virus by propagation in chicken embryos or in cell culture using primary chicken embryo cells (CEC), primary chicken embryo fibroblast cells (CEF), or cell lines such as Quail derived cells (QT35, QM5, or QM7) or mammalian cells (Vero cells). Adaptation of wild-type IBDV is

always reported to correlate with attenuation (32, 34). Adapted IBDV is able to infect non B-lymphoid chicken cells, resulting most likely in a reduced viral load in the B-lymphoid cells in the bursa of infected chickens. Several reports have appeared
5 which described amino acid mutations resulting from adaptation of wild-type IBDV during propagation on non B-lymphoid cells (20, 33, 34). Furthermore, two studies have been published in which amino acid mutations have been introduced, using a reverse genetics system, in the VP2 region of IBDV (20, 22).
10 These analyses show that important amino acids for propagation on non B-lymphoid cells are found within the hypervariable region of VP2 (i.e. amino acids at position 253, 279, and 284). The influence of mutations found in other parts of the genome (e.g in VP1 (7, 34)) is unclear at the moment. Studies focussed
15 at determining the influence of site directed single or multiple amino acid mutations are hampered by the lack of a reverse genetics system which can generate very virulent IBDV. In this report we describe such a system. Using the full-length cDNA of a wild-type very virulent IBDV isolate (D6948) we have
20 successfully rescued recombinant D6948 (rD6948). Furthermore we rescued mosaic IBDV after transfection of plasmids which contained largely cDNA originating from an attenuated classical isolate (CEF94) and partly of cDNA originating from wild-type vvIBDV (D6948).

25

Material and Method

Viruses, cells, and antibodies. The classical IBDV isolate CEF94 is a derivative of PV1 which has been adapted for
30 growth on cell cultures. (3, 23). The wild-type vvIBDV isolate D6948 was originally isolated by the Poultry Health Service of the Netherlands (PHD, Doorn, 1989) and was purified by repeated limited dilution passages in embryonated eggs (5 times). It was subsequently passed twice in SPF chickens in our laboratory.
35 Recombinant Fowlpox virus containing the T7 polymerase gene (FPV-T7) (6) was received from the laboratory of M. Skinner (Compton Laboratory, Berks, United Kingdom). QM5 cells (1) were maintained by using QT35 medium (Gibco-BRL), supplemented with

5% Fetal Calf Serum (FCS) and 2% antibiotics solution ABII (1000 U/ml Penicillin (Yamanouchi), 1 mg/ml Streptomycin (Radiumfarma, Italy), 20 mg/ml Fungizone, 500 mg/ml; Polymixin B, and 10 mg/ml Kanamycin), in a CO₂ (5%) incubator at 37° C.

5 Primary bursa cells were isolated from 14-days old SPF embryos and were maintained in Eagle's modified Minimal Essential Medium (EMEM) supplemented with 15% FCS, 0.125% Lacto Albumine Hydrolysate (Oxiod) 1000 Units Penicillin/ml (Yamanouchi) and 1 mg/ml Streptomycin (Radiumfarma, Italy). Polyclonal rabbit
10 antiserum against VP1 was produced by injecting rabbits with either purified recombinant VP1 (30). A Polyclonal rabbit serum against VP3 and VP4 of IBDV was produced as follows: A PCR fragment containing nt 2297 to nt 3192 of the A-segment cDNA (amino acids 722 -> 1018 of the polyprotein) of CEF94 was fused
15 to a his (6*) tag, using *E. coli* expression plasmid pQE-30 (Qiagen). The expressed fusion protein was purified using Ni-NTA resin (Qiagen) according to the suppliers instruction. The purified proteins were separated in a SDS-PAGE gel and full length fusion product (+/- 35 kDa) was isolated from the gel
20 according to Hardy et al. (16). The recovered protein was subsequently used to immunize rabbits. The specificity (both VP4 and VP3 are recognized) and reactivity of the rabbit serum (PaVP3/4) was confirmed in Elisa, Radio-Immune-Precipitation, and IPMA analysis (data not shown).

25

Generation of full length A- and B-segment clones. To produce full-length single stranded cDNA of both the A- and B-segments of the two IBDV isolates (CEF94 and D6948) we used
30 primers specific for the 3'end of the coding strand for reverse transcription (4). Two primers specific for the 3'-end of both the coding and non-coding strand were subsequently used to amplify the full-length A- and B-segment cDNAs in a PCR amplification using a mixture of Taq and Pwo enzymes (Expand,
35 Boehringer Mannheim) (4). The two primers which hybridize with the 3'-terminus of the non-coding strand contained a 5' extension encoding the T7 promotor (4). Three independent RT-PCR reactions were performed for each segment and the resulting

PCR fragments were cloned into the pGEM-T vector (Promega) (A-segment) or in a pUC19 derivative which contained the antigenomic cis-acting Hepatitis Delta Virus (HDV) ribozyme (10) and a T7 polymerase terminator (see Fig. 17 and (3) (B-segment). Sequence analysis was performed on both strands using sequence specific primers in a cycle sequencing reaction (BigDye terminator kit, PE Applied Biosystems) and an ABI310 apparatus (PE Applied Biosystems). An unintended mutation in one of the A-segment of D6948 clones (pHB-22), was restored by exchanging a restriction enzyme fragment from an independent clone (data not shown), yielding pHB-22R. pHB-22R contains the consensus cDNA of the A-segment of the D6948 vvIBDV isolate. We subsequently transferred this full-length A-segment sequence into a pUC18-based vector, which contained the Hepatitis Delta Virus (HDV) ribozyme and a T7 polymerase terminator (see above) by PCR amplification using the same primers as used in the RT-PCR protocol (4). During this transfer an unintended mutation was introduced in the VP4 encoding part of the polyprotein (A1817G). This mutation was subsequently used as a genetic tag for virus rescued from this D6948 A-segment plasmid. The A-segment cDNA clone of CEF94 (pHB-36W) contains a two nucleotid genetic tag (3172C->T and 3173T->A) thereby introducing a unique *KpnI* restriction site GGTAAC in the 3'-UTR of the A-segment (3).

Protein sequence comparisons. Amino acid sequences used for sequence alignments were retrieved from the GenBank database (accession numbers are given between parentheses). For the alignment of VP5 and polyprotein (A-segment) of the vvIBDV we used the predicted amino acid sequence of isolates D6948 (AF240686), HK46 (AF092944), UK661 (X92760), and OKYM (D49707). For the alignment of the vvIBDV VP1 sequences (B-segment) we used the predicted amino acid sequence of D6948 (AF240687), HK46 (AF092944), UK661, (X92761), OKYM (D49707), IL3 (AF083093), and IL4 (AF083092).

In vitro transcription and translation. Circular plasmids (0.4 mg) containing full-length A- or B-segments preceded by a T7-promotor were used as template in a 2.5 ml in vitro transcription-translation reaction (TnT-T7Quick, Promega) in the presence of ³⁵S-Methionine. The resulting viral proteins were separated in an SDS-PAGE gel (12%), and visualized by autoradiography.

Infection and transfection of QM5 cells. QM5 cells were grown to 80% confluency in a 35 mm culture dish (M-6, 9.6 cm²/well) and infected with Fowlpox virus-T7 (FPV-T7, m.o.i. = 3). After one hour, the cells were washed twice with 3 ml of QT-35 medium and covered with 3 ml Optimem 1 (Gibco/BRL). In the mean time, DNA (1.0 mg) was mixed with 12.5 ml LipofectAMINE (Gibco/BRL) in 250 ml Optimem 1 and kept at room temperature for at least 30 min. The QM5 cells were subsequently covered with 2 ml of fresh Optimem 1 and the DNA/LipofectAMINE mixture was added. The transfection was performed overnight (18h) in a 37° C incubator (5.0% CO₂). The transfected monolayer was rinsed once with PBS, and fresh QM5 medium (supplemented with 5% FCS and 2% ABII) was added and the plates were further incubated in the 37° C (5.0% CO₂) incubator. After 24h of incubation the plates were freeze/thawed once and the supernatant was filtered through a 200 nm filter (Acrodisc, GelmanSciences) to remove the Fowlpox virus (FP-T7) and cellular debris. The cleared supernatant was either stored at -70° C or used directly for further analysis.

Detection of infectious rIBDV. After transfection, infectious rIBDV was detected by inoculating a near confluent monolayer of QM5 cells with part of the cleared transfection supernatant. IBDV specific proteins were visualized after 24h in an Immuno-Peroxidase Monolayer Assay (IPMA) (3). Rescued IBDV (e.g. rD6948) which is unable to replicate on QM5 cells was also used to inoculate a monolayer of primary bursa cells which had been grown in vitro for 24h in a 35 mm tissue culture dish (10 cm²) in a CO₂ incubator (5%) at 39° C. IBDV specific

proteins in infected B-lymphoid cells were detected in an IPMA after 48h.

Single step IBDV growth curves. To assess the replication ability of the rCEF94 and srIBDV-CADB isolates (see Table 16) we determined single step growth curves. QM5 cells (2×10^6) were grown overnight (16 h) in a 60 mm cell culture dish. The medium was subsequently removed and 1 ml medium containing IBDV ($TCID_{50} = 10^{7.0}$; m.o.i. = 5) was used to cover the cells. After one hour the medium was removed, the cells were rinsed three times with PBS, and 5 ml fresh medium was added. At different time points (5h, 10h, 15h, and 25h post infection) samples were taken from the medium and stored at -20°C . The amount of IBDV ($TCID_{50}$) in each sample was determined by infecting 96-well tissue culture plates, containing near confluent QM5 cells, with 10-fold dilutions of the IBDV samples. The 96-well plates were incubated for 48 hours, and infective centers of IBDV (IBDV-antigen positive areas) were detected by an IPMA (see Detection of infectious rIBDV).

Construction of mosaic A-segment plasmids pHB36-vvVP2. To replace the coding sequence of the pVP2 part of the CEF94 polyprotein with the corresponding part of the D6948 polyprotein we generated three PCR fragments (i.e. VP2a, VP2b and VP2c, see Fig. 21). PCR fragment VP2a (189 bp) was generated using primers M13R (TCACACAGGAAACAG CTATGAC) and ATG3 (CATCGCTGCGATCGTTTGTCTGATCTCTAC), and pHB-36W as template. PCR fragment VP2b (761 bp) was generated by using primers (ATCCGGGCCCTAAGGAGG) and ANC4 (GCCAAGTCGGTGTGCAG), and pHB-36W as template. PCR fragment VP2c (1418 bp) was generated using primers HY0P (TATCATTGATGGTCAGTAGAG) and HY2M (CACCGGCACAGCTATCC), and pHB-22R as template. These three PCR fragments were agarose gel purified using Qiaex gel extraction kit (Qiagen, Germany) and used as template (50 ng of each fragment) in a fusion PCR using primers T7EcoRI (GGAATTCTAATACGACTCACTATAGG) and ANC4. This PCR fragment (2256 bp) was subsequently digested with EcoRI and SacII (resulting

in a 1806 bp fragment), agarose gel purified (Qiaex gel extraction kit, Qiagen) and ligated into pHB-36W, which had been digested with the same restriction enzymes, and was then used to transform *E.coli* DH5a cells. Several plasmids were
5 analyzed and one plasmid having the intended sequence was selected (pHB36-vvVP2).

Construction of mosaic A-segment plasmids pHB36-vvVP3. To replace the coding sequence of the VP3 part of the CEF94
10 polyprotein with the corresponding part of the D6948 polyprotein we generated two PCR fragments. PCR fragment VP3a (1622 bp) was generated using primers AC3 (GGTAGCCACATGTGACAG) and HY3M (CCAGTCCCGcGGATTGTGAGG), and pHB-36W as template. PCR
15 fragment VP3b (1252 bp) was generated by using primers HY3P (AACGTTTTCTCCTACAATCCgCGgGACTGGG) and M13F (GTAAACGACGGCCAGT) and pHB-22R as template. These two PCR fragments were agarose gel purified and used as template (50 ng of each fragment) in a fusion PCR using primers AC4 (ACCCAGCCAATCACATCC) and AGTM (GAGACTCCAGGtaCCTCACT). This PCR fragment (2154 bp) was
20 subsequently digested with *EagI* and *KpnI* (resulting in a 1857 bp fragment), and used to exchange the corresponding part of pHB-36W in the same way as described for pHB36-vvVP2, yielding pHB36-vvVP3.

Construction of mosaic A-segment plasmids pHB36-vvVP4. To replace the coding sequence of the VP4 part of the CEF94
25 polyprotein with the corresponding part of the D6948 polyprotein we generated three PCR fragments. PCR fragment VP4a (796 bp) was generated using primers AC3 and HY4M (CCGGCACAGCTATCCT), and pHB-36W as template. PCR fragment VP4b (644 bp) was generated by using primers HY3P and ANC2 (CTGCCTGTCCTGGAGCC), and pHB-36W as template. PCR fragment VP4c (864 bp) was generated using primers HY4P (ACATAATCCGGGCCATAAGG) and HY3M, and pHB-22R as template. These
30 three PCR fragments were agarose gel purified and used as template (50 ng of each fragment) in a fusion PCR using primers AC4 and ANC3 (CGATGGGCGTTCGGGTC). This PCR fragment (2154 bp) was subsequently digested with *EagI* and *DraIII* (resulting in a

1189 bp fragment), and used to exchange the corresponding part of pHB-36W in the same way as described for pHB36-vvVP2, yielding pHB36-vvVP4.

- 5 **Virulence of rIBDV in young SPF chickens.** The virulence of the rescued rIBDV, srIBDV, and mIBDV (see Table 16) isolates was evaluated in SPF layer-type chickens. The IBDV isolates were first propagated on embryonated eggs, by inoculating the QM5 cell supernatant of a transfection experiment into 11-days
- 10 old embryonated eggs via the chorioallantoic membrane route. After five days of incubation the embryos (dead or alive) were recovered, homogenized in a Sorval Omni-mixer (3 * 10 sec, max. speed) and subsequently clarified by centrifugation (6000 g, 10 min.), and stored in aliquots at -70° C. The virus titers (50%
- 15 embryo lethal dose, ELD₅₀) in these samples were determined using 11-days old embryonated eggs. In the virulence test, 9 groups of chickens (21-days old) were inoculated intranasally and by eye-drop with 1000 ELD₅₀ IBDV in PBS. In addition one group of 10 chickens received PBS only (negative control
- 20 group). The groups of chickens were housed separately in isolators. The animals were monitored for clinical signs daily and dead chicks were removed and necropsied. At 9 days post infection (p.i.), all chicks from the negative control groups and all remaining chicks from groups in which mortality had
- 25 occurred, were bled (5 ml) and euthanized for necropsy. From the other groups, 6 chicks were bled (5 ml) and taken for necropsy at day 9 p.i., whereas the remaining 4 were bled (5 ml) and taken for necropsy at day 15 p.i.. Bursa and body weight were determined of all chicks that had been euthanized
- 30 at day 9 p.i.. The bursa/body weight ratios were analyzed by a one way analysis of variance with a factor for groups. Ratios were log-transformed prior to analysis. Pairwise comparisons between groups were based on Fisher's LSD method (pairwise t-tests with a pooled variance estimator). At necropsy at day 9
- 35 and 15 p.i. samples from the bursa of Fabricius were fixed in 10% neutral-buffered formalin for histopathology. Bursa samples were snap frozen in liquid nitrogen and preserved at -70° C for immunohistochemical examination. IBDV antibody titers in serum,

taken just before euthanasia, were determined using the Infectious Bursal Disease Antibody detection kit (Idexx, Westbrook, USA).

5

Histopathology and immunohistochemistry. Formalin fixed bursa samples were dehydrated, embedded in paraffin wax, sectioned and stained with Hematoxylin-Eosin (H & E).

- 10 Histopathologic bursal lesion score (HBLS) was determined by microscopic analysis of the bursa. HBLS was determined on a scale of 1 to 5 according to (2): 1 = normal bursa; 2 = scattered or partial follicle damage; 3 = 50% or less follicle damage; 4 = 50-75% follicle damage; 5 = 75-100% follicle damage. Frozen bursa samples were sectioned for
- 15 immunohistology on a cryostat at 8 μ m thickness and taken up on glass slides (Superfrost®). The sections were fixed with acetone for 10 min, air-dried and stored at -20 °C until used. An immunoperoxidase staining was performed as described (25). Briefly, endogenous peroxidase in
- 20 sections was eliminated by 0.01% H₂O₂. Non-specific reactions were blocked by 0.2% bovine serum albumin. A 1:100 dilution of PaVP3/4 was used as the primary antibody, whereas a 1:100 dilution of goat anti-rabbit IgG antibody conjugated to peroxidase (Dako) was used as
- 25 the secondary antibody, and Diamino-benzidine (DAB) as substrate.

- Detection of genetic tag.** Bursae of chickens originating
- 30 from the groups in which mortality had occurred during the animal experiment (see above) were homogenized and viral dsRNA was extracted using a QiaAmp Tissue Kit (Qiagen, Germany). The dsRNA was concentrated by standard ethanol precipitation and subsequently used as template in a reverse transcription
- 35 reaction using primer ANC1 (GGGGACCCGCGAACG). A PCR (Taq polymerase) was performed on this template using primers AC3 and ANC5 (CCCATCTGGAGCATATCC) and cycling 35 times between 94° C (15 s), 55° C (15 s), and 72° C (90 s). The obtained PCR

products (1266 bp) were gel purified (Qiaex gel extraction kit, Qiagen), and used as template for sequence analysis, by using primer AC6 (TTCACCTGGGGTACTCCG).

5 Results

Cloning of full-length cDNA of vvIBDV. To study molecular determinants that are responsible for the very virulent phenotype of vvIBDV isolates, we cloned and sequenced the full-length A- and B-segments of the very virulent IBDV isolate D6948 three times independently. The cDNA of the D6948 A-segment (3260 bp) differs at 122 positions from the sequence of the classical, attenuated CEF94 IBDV isolate (data not shown). These nucleotide differences result in an N-terminal extension of 4 amino acids (MLSL) for the predicted VP5 of D6948, and 5 additional amino acid differences between the two VP5 proteins (Fig. 16a). Furthermore, 18 amino acid differences are present between the two polyproteins, of which 11 are located in the pVP2 part, 5 in the VP4 part, and 2 in the VP3 part of the polyprotein (Fig. 16b). Between the two B-segments (2827 bp) we found 288 nucleotide differences (data not shown), resulting in 17 amino acid differences between the two VP1's (Fig. 16c), the only protein encoded on the B-segment.

By combining sequences of different full-length cDNA clones we constructed plasmids which contained the consensus cDNA sequence of the D6948 A- and B-segment. The A- and B-segment cDNAs, including an artificially introduced T7 promotor sequence, were subsequently transferred to a pUC19 based vector which contained a cis-acting hepatitis delta virus ribozyme (10), yielding pHB-60 (A-segment) and pHB-55 (B-segment) (Table 16, and Fig. 17). These pUC-19 based transcription plasmids are the basis for rescuing rIBDV from cloned cDNA, using a recombinant helper virus (Fowlpox) that expresses T7 polymerase (6). We have used this *in vivo* T7 expression system previously to rescue infectious IBDV from cloned cDNA of the cell culture adapted, classical IBDV isolate CEF94 (3).

In vitro transcription/translation. The A- and B-segment cDNA clones of vvIBDV isolate D6948 were used as templates in an *in vitro* transcription/translation reaction (TnT-T7Quick, Promega). The protein products of these cDNA clones appeared to be identical to those of the classical attenuated CEF94 cDNA plasmids (i.e. pVP2, VP3, and VP4 in case of the A-segment and VP1 in case of the B-segment) in an SDS-PAGE analysis (Fig. 18A and B). The pVP2 from D6948 is found at a slightly higher position as the pVP2 protein from CEF94. Plasmid pHB-60 (D6948 A-segment) contains a single nucleotide substitution at position 1817 nt (A1817G), resulting from the PCR-based cloning strategy. This mutation leads to a conserved amino acid substitution in VP4 (I563V). As this mutation did not affect the processing of the polyprotein (Fig. 18A, and unpublished results), we have subsequently used this mutation as a tag for virus rescued from this D6948 A-segment plasmid.

Transfection of QM5 cells. Plasmids containing the A- and B-segment cDNA of either CEF94 or D6948 were used to co-transfect QM5 cells. Transient expression of the viral proteins originating from both the A- or B-segment was observed in all examined cases in an immunoperoxidase monolayer assay (IPMA), by using antibodies specific for either VP3/4 (A-segment) or VP1 (B-segment) (data not shown). To assess the production of infectious IBDV originating from cloned cDNA (referred hereafter as rescued IBDV; rIBDV) we transferred part of the supernatant onto a fresh monolayer of QM5 cells (first passage) and analyzed the expression of viral proteins in these cells after 24h in an IPMA. This analysis showed that no infectious rIBDV could be detected in QM5 cells treated with the supernatant of a co-transfection of the A- and B-segments plasmids of vvIBDV isolate D6948 (see Fig. 19). In contrast, infectious rIBDV was present in QM5 cells treated with supernatant of the co-transfection with plasmids containing the A- and B-segment of the attenuated CEF94 isolate (Fig. 19). The co-transfection of the A-segment plasmid of D6948 with the B-segment plasmid of CEF94 yielded no infectious segment reassorted IBDV (srIBDV-DACB, Table 16). However, the

reciprocal combination srIBDV-CADB (Table 16) did yield infectious segment reassorted IBDV (Fig. 19). Wild-type IBDV isolates such as D6948 are unable to grow on fibroblast cells like QM5 (see Introduction). Therefore we assessed the presence of infectious rD6948 originating from cloned cDNA by transferring supernatants of transfected QM5 cells onto primary bursa cells which had been grown in vitro for 24h. Similar to wild-type D6948, which can infect only the lymphoid cells present in this monolayer (data not shown), we were able to detect rD6948 in several lymphoid cells after two days of incubation with the transfection supernatant (Fig. 19, lower panel). Incubation of primary bursa cells with the transfection supernatant of A-and B-segment cDNAs from CEF94 showed that not only lymphoid cells but also fibroblast cells were infected, leading to a destruction of the monolayer within 48h of incubation. Virus present in the supernatant of a transfection with the A-segment cDNA of D6948 and B-segment cDNA of CEF94 was only able to infect lymphoid cells, and no destruction of the monolayer was observed after 48h. Similar to rD6948, srIBDV-DACB is apparently unable to infect fibroblast cells. Again, infection of fibroblast cells was found with virus originating from transfection of the A-segment cDNA of CEF94 and B-segment cDNA of D6948, although the monolayer appeared to be less destructed after 48h in comparison with rCEF94 (Fig. 19).

Single step growth curves. To determine whether the rescued rCEF94 and srIBDV-CADB had the same replication properties on QM5 cells as wild-type CEF94, we performed single step growth curves (in triplicate) for each of these viruses. QM5 cells were infected during one hour with IBDV (m.o.i. = 5), after which time the cells were rinsed three times and incubated in complete medium. Part of the supernatant of the infected QM5 cultures was removed at different time points. The amount of IBDV (TCID₅₀ per ml) was determined in each sample (Fig. 20). Release of infectious IBDV was found at 10h p.i. for both wild-type CEF94 and rCEF94, while the first release of srIBDV-CADB was only found at 15h p.i.. Final titers (24h p.i.) were about

the same for wild-type CEF94, rCEF94, and the rescued segment reassorted IBDV containing the A-segment of CEF94.

Construction of mosaic A-segment plasmids. To analyze the viral determinants responsible for the very virulent phenotype of isolate D6948, we constructed three different plasmids containing full-length mosaic IBDV A-segment cDNA. Using a PCR based method (see Fig. 21) we constructed DNA fragments of which the middle part consisted of cDNA originating from D6948 (i.e. the pVP2, VP3 or VP4 encoding part), while the flanking cDNA originated from CEF94. The transition positions between the different cDNAs are at the putative cleavage sites between the pVP2-VP4 and VP4-VP3 parts of the polyprotein (i.e. behind amino acid 453 and 723, respectively (18), see Fig. 16B). The mosaic PCR fragments were subsequently used to replace the corresponding part of CEF94 A-segment cDNA in plasmid pHB-36W, using specific restriction endonucleases. The exchanged parts of the generated plasmids, named pHB36-vvVP2, -vvVP3, and -vvVP4, were sequenced and plasmids containing the intended mosaic cDNA sequence were subsequently used for *in vitro* transcription/translation and analyzed for the production of viral proteins by means of SDS-PAGE (Fig. 18C). No apparent difference in the autocatalytic cleavage was observed in the polyproteins encoded by these mosaic cDNA A-segments.

Rescue of IBDV from mosaic A-segment cDNA. The plasmids pHB36-vvVP2, -vvVP3, and -vvVP4 were used to transfect QM5 cells together with the B-segment cDNA of CEF94 (pHB-34Z). The presence of infectious IBDV (hereafter referred to as mIBDV, see also Table 16) was analyzed by transferring aliquots of the transfection supernatant to a fresh monolayer of QM5 cells. After 1 day of incubation we fixed the cells and used an IBDV specific antibody assay (IPMA) to test for the presence of mIBDV. No mIBDV could be detected in the QM5 monolayer when supernatant of the pHB36-vvVP2 plasmid was used while in the case of pHB36-vvVP3, and pHB36-vvVP4, mIBDV was clearly present (Fig. 22). The fact that we did not detect mCEF94-vvVP2 in the

QM5 monolayer could result from the difference in cell tropism between the rCEF94 and mCEF94-vvVP2. To check for this possibility we transferred part of the supernatant of the transfection with pHB36-vvVP2 onto a monolayer of primary bursa cells. After two days we were able to show the presence of mCEF94-vvVP2 virus in some of the B-lymphoid cells (Fig. 22, lower panel). Apparently, mCEF94-vvVP2 had the same cell-tropism as D6948, rD6948, and srIBDV-DACB, since it was only able to grow on B-lymphoid cells and not on fibroblast cells.

10

Animal experiment. To compare the virulence of rIBDV, srIBDV and mIBDV isolates with the parent isolates, we inoculated 21-day-old SPF chickens (10 chickens per virus) intranasally and by eye-drop in one eye with 1000 ELD₅₀ each. Clinical signs occurred only in the groups that had received D6948, rD6948 and srIBDV-DACB (data not shown). In these groups the mortality was 3 out of 10 (D6948), 5 out of 10 (rD6948), and 2 out of 10 (srIBDV-DACB) (Table 17). Neither morbidity nor mortality was found in the other groups. At day 9 p.i. all remaining chickens of the groups in which mortality had occurred (D6948 (n=7), rD6948 (n=5) and srIBDV-DACB (n=8)), and all chickens from the control group were bled and necropsied. From the remaining groups 6 chickens were bled and necropsied, while the remaining 4 were left until 15 days p.i. to determine the IBDV antibodies level. The bursa/body weight ratio in all groups in which mortality occurred was very much reduced (< 2.0). Fisher's LDS test showed that the bursa/body weight ratios of D6948, rD6948, srIBDV-DACB, and mCEF94-vvVP2 are indeed significantly lower ($P < 0.01$) than the values of the control group and of the groups which received the other viruses. That reduction in bursa/body weight ratio does not always correlate with the occurrence of mortality is clear from inoculation with mCEF-vvVP2. Although the bursa/body weight ratio is severely reduced (< 2.0), no morbidity (data not shown) or mortality (Table 17) occurred in this group. The mCEF94-vvVP3 and -vvVP4 isolates induced neither mortality, nor morbidity nor a large reduction in the bursa/body weight ratio. Only in those groups where the bursa/body weight ratio was

severely reduced, we found that all chicks possessed antibodies against IBDV 9 days p.i., while several chickens possessed no IBDV antibodies in the groups where no mortality had occurred (Table 17). All birds which were examined at 15 days p.i. possessed IBDV antibodies, except for 3 chickens (of the 4 examined) which had received wild-type CEF94 virus (data not shown).

Detection of genetic tag. The bursae of several chickens of the groups in which mortality had occurred (D6948, rD6948, and srIBDV-DACB) were recovered. Viral RNA derived from the homogenized bursae was used as template in an RT-PCR (see Materials and Methods section). The resulting PCR fragments, spanning the A-segment from nt 709 to nt 1975, were used for the nucleotide sequence determination. The PCR fragments derived from the groups inoculated with rD6948 and srIBDV-DACB contained the genetic tag (A1817G), which was present in the A-segment cDNA plasmid (pHB-60) (data not shown). The PCR-fragment originating from dsRNA of bursae infected with wild-type D6948 did not contain this genetic tag (data not shown).

Several studies have been published in which amino acid changes of (very) virulent IBDV isolates, resulting from adaptation to non B-lymphoid cell cultures, have been mapped (20, 22, 33). The reverse genetic IBDV systems described so far (3, 20, 22) are only capable of rescuing IBDV from cloned cDNA originating from adapted (i.e. being able to grow on non B-lymphoid cell) IBDV isolates. As a result of this limitation no mutagenesis studies can be performed in rescued IBDV possessing the original B-lymphoid cell tropism. To overcome this limitation, we have modified our method of rescuing IBDV from cloned cDNA.

The possibility was explored of using primary bursa cells for rescuing vvIBDV. Therefore we first transfected primary bursa cells with the A- and B-segment cDNA containing plasmids. We found that transfection of B-lymphoid bursa cells was very inefficient. The cells are very fragile and both the isolation and maintenance of these B-lymphoid cells is laborious.

Furthermore, fibroblast cells, isolated from the bursa along with the B-lymphoid cells, grow rapidly *in vitro*, in contrast to the lymphoid cells. This difference in growth rate results in a monolayer consisting mostly of non-B-lymphoid cells after
5 42h of incubation, the time required for transfection using the Fowlpox T7 *in vivo* expression system.

In search for a more efficient system of rescuing vvIBDV, we modified subsequently our method of rescuing cell culture adapted IBDV (CEF94, (3)) from transfected QM5
10 cells by harvesting supernatant of transfected QM5 cells (non-permissive for propagation of wild-type IBDV), and transferring this to a monolayer of primary bursa cells (containing B-lymphoid cells which are permissive for propagation of wild-type IBDV). This method allowed us to
15 rescue vvIBDV from its cloned cDNA (rD6948). Rescued D6948 was subsequently used in an animal experiment, along with wild-type D6948 to assess its virulence. Chicks (21-day-old, SPF) were infected with different IBDV isolates. Comparison of all analyzed parameters (mortality, bursa/body weight
20 ratio, histopathologic bursa lesion score, immunohistochemistry, antibody titers (Table 17), and morbidity (data not shown)) collected during 9 days p.i. showed an identical virulence for wild-type and rescued D6948. This showed that the rescued D6948 is indeed very
25 virulent. The dsRNA of both D6948 and rD6948 was isolated from the bursa of infected chickens and used in a RT-PCR, followed by direct sequence determination. The genetic tag in the VP4 part of the polyprotein encoded by the D6948 A-segment cDNA plasmid was as expected present in rD6948
30 derived dsRNA (data not shown). The single mutation in VP4 (I563V), which results from the genetic tag in the A-segment cDNA does not result in a difference in phenotype (*in vitro* and *in vivo*) of rD6948, compared to D6948. This is the first report describing the rescue of a wild-type vvIBDV isolate
35 from cloned cDNA.

After having verified that the deduced cDNA sequence of D6948 represents indeed a true vvIBDV sequence, we compared the deduced amino acid sequence of D6948 proteins with those of other (vv)IBVD isolates. One striking difference between the

5 VP5 sequences of the classical attenuated isolate CEF94 and the vvIBDV isolate D6948 is that the vvIBDV VP5 has an N-terminal extension of 4 amino acids (see Fig. 16a). This extension is also predicted for the two other vvIBDV isolates of which the sequences of this region have been published (HK46 (20) and

10 UK661 (7)). All classical isolates lack this N-terminal extension, while the only antigenic variant (GLS) of which the cDNA sequence in this region is available has the same extension. Whether the AUG-codon at position 85 is indeed used as a startcodon for the production of VP5 of the very virulent

15 and the GLS isolate remains to be determined. It would be interesting to see if the N-terminal extended VP5 results in a different phenotype. Of the other amino acid differences in VP5 (Fig. 16A), only R49G and W137R are found for all known vvIBDV VP5 sequences (UK661, OKYM (33), and HK46. Several reports have

20 focused on differences between the pVP2 sequences of IBVD isolates of different origin (e.g. (15)). The amino acid difference at position 253, 279, 284, and 330 (Fig. 16B) are most likely the result of adaptation of CEF94 on CEF cells (20, 22, 33). Of the remaining amino acid differences only 8 are

25 conserved (see amino acid residues in bold face, Fig. 16B) among all the four vvIBDV isolates (D6948, UK661, OKYM, and HK46) in compared with the published sequences of classical and antigenic variant isolates.

30

The length of the ORF for VP1/VPg of CEF94 is two amino acids longer than the same ORF of D6948. Heterogeneity in the length of the VP1/VPg ORF has also been found by others (34). All VP1/VPg's of vvIBDV

35 isolates appear to have a size of 879 amino acids, while some of the VP1's of classical isolates have a size of only 877 amino acids. The 881 amino acids found for the VP1/VPg of CEF94 is the largest VP1 published so far. Furthermore, we identified 17 amino acid differences

between the VP1/VPg sequences of CEF94 and D6948 (Fig. 16C). Although it has been found that attenuation of IBDV results in amino acid changes in the VP1 sequence (34), no direct correlation between the three changes identified by Yehuda et al. and attenuation has been proven. In fact we show that segment reassorted IBDV (srIBDV-DACB, see Table 16) which contains the B-segment of a classical, attenuated IBDV isolate, induces the same mortality and histopathologic lesions as wild-type very virulent IBDV. This indicates that the amino acid changes found in VP1 after adaptation are not related to the attenuation of the virus. Six of the 17 amino acid differences are found in all of the vvIBDV isolates of which the cDNA sequence has been published (see amino acid residues in bold face in Fig. 16C).

To assess the influence of the amino acid differences between the viral proteins from different isolates we constructed plasmids which contained cDNA from both CEF94 and D6948. A small difference in molecular weight between the pVP2 proteins encoded by the cDNA of CEF94 and D6948 was found in an SDS-PAGE analysis (Fig. 18A). The difference in position in the SDS-PAGE gel is also found for the pVP2 proteins encoded by pHB36W and pHB36-vvVP2 (Fig. 18C). A small difference is present between the position of VP4 encoded by pHB36-vvVP3 and pHB-36-vvVP4, in comparison with VP4 encoded by pHB-36W. The first amino acid difference between the two VP3 proteins of CEF94 and D6948 (H751D, Fig. 16B) is found 28 amino acids downstream of the proposed cleavage site (behind amino acid 722 (18), see Fig. 16B). The difference in the molecular weight of VP4 can be explained if the actual cleavage site of VP4-VP3 is not behind the two basic amino acid residues, but at least >28 amino acids more downstream. Alternative cleavage sites between VP4/VP3 have been proposed to be located behind amino acid 736 (after Tyr-Leu (13) or in the region 752-756 (A-X-A-A-S, (18))). Recently it was indeed shown, using a site-directed mutagenesis approach, that the cleavage site between VP4 and VP3 is most likely located between amino acid residues 755 and 756 (27). We

observed that polyclonal antibodies raised against part of the polyprotein starting at position 722 is also able to react strongly with VP4, adding further prove to the assumption that the actual cleavage site is located downstream of amino acid 722 (Boot unpublished data).

The viruses derived from the mosaic A-segment plasmids were checked for growth on either QM5 cells and/or primary bursa cells and used to challenge SPF chickens. Segment reassorted virus containing the A-segment of D6948 and the B-segment of CEF94 (srIBDV-DACB, Table 16) induced the same signs and lesions as wild-type and recombinant D6948 (Table 17). The differences found within the VP1/VPg proteins of classical (adapted) and very virulent (non-adapted) isolates have apparently no major influence on virulence. The reciprocal combination (srIBDV-DACB, Table 16) induced less CPE in the monolayer of primary bursa cells in comparison to rCEF94 (Fig. 19). We also found a delay in release of new viral particles by srIBDV-CADB in comparison with CEF94 and rCEF94 (Fig. 20). Despite the difference in *in vitro* virus release between these isolates, we found no difference in the *in vivo* challenge using SPF chickens. IBDV antibody titers of rCEF94 and srIBDV-CADB are comparable both at 9 days p.i. (Table 17) and 15 days p.i. (data not shown). It has been suggested that segment reassortment between B-segments of serotype I and II isolates occurs in the field (7). Natural occurring segment reassorting is frequently found for highly segmented dsRNA viruses (Reo- and Rotavirus, (26)). We did not find any phylogenetic evidence for a natural reassortment of IBDV segments. All VP1 proteins of vvIBDV have distinct amino acid changes, when compared to classical serotype I isolates and serotype II isolates (Fig. 16C, and data not shown). Although no prove exists for IBDV reassortments in the field, Müller has succeeded to produce segment reassorted virus in the laboratory by co-cultivation of strains belonging to the different serotypes (21).

Exchange of either pVP2 (including a large part of VP5), VP3 or VP4 of CEF94 with the corresponding proteins of

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D6948 yielded in all cases viable virus. Both the mCEF94-vvVP3 and mCEF94-vvVP4 are able to replicate on QM5 cells, while mCEF94-vvVP2 is only able to replicate on B-lymphoid cells. No differences were found in the single step growth curves of mCEF94-vvVP3, mCEF94-vvVP4, CEF94 and rCEF94 on QM5 cells (data not shown). It has been shown previously that the factor responsible for propagation in non-B-Lymphoid cells is located on VP2 (20, 22, 32). In this study we show that wild-type vvIBDV is able to replicate in QM5 cells once it is artificially (transfection of cDNA) introduced in these cells. Thus the inability to replicate on non-B-lymphoid cells is apparently located in the inability to recognize the receptor and/or the inability to enter the cell. Two possible explanations for the this change in cell tropism, resulting from mutations in wild-type VP2, exist. 1) These mutations lead to a (conformational) change in VP2, resulting in recognition of a receptor present on a wide range of cells (general IBDV receptor) and, at the same time, loss of recognition of the specific B-lymphoid cell receptor (B-lymphoid IBDV receptor). The domain responsible for interacting with the general IBDV receptor and with the B-lymphoid IBDV receptor is in this case located in the same region of VP2 (e.g. the hypervariable region). 2) These mutations lead to a (conformational) change in VP2, resulting in recognition of the general IBDV receptor, but leaves the recognition of the B-lymphoid IBDV receptor (either by VP2, VP3, or a combination of both) intact. The phenomenon that cell culture adapted IBDV is still able to enter and replicate in B-lymphoid cells is explained by the assumption that the general IBDV receptor is also present on B-lymphoid cells (entry model 1 and 2). Entry model 2 even predicts replication of cell culture adapted isolates in B-lymphoid cells when the general IBDV receptor is missing on B-lymphoid cells, by usage of the genuine B-lymphoid IBDV receptor.

It has been found that two specific amino acid mutations (Aspartic acid²⁷⁹ to Asparagine²⁷⁹, and Alanine²⁸⁴ to Threonine²⁸⁴) alone, or in combination with a third

- mutation (Glutamine²⁵³ to Histidine²⁵³) might be enough to alter the tropism of an wild-type IBDV strain from an exclusive B-lymphoid cells tropism into a more general tropism (e.g. VERO, QM7, QM5 or CEF cells). Only limited
- 5 data (20, 22, 33) has been published supporting this. It might be possible that a IBDV strain which possess all these three adaptation mutations (Histidine at position 253, Asparagine at position 279 and Threonine at position 284) is able to grow on B-lymphoid cells, but is still
- 10 unable to grow on non B-lymphoid cells, due to a specific amino acid sequence somewhere else in VP2 or in one of the other viral proteins.

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LEGENDS TO FIGURES

Fig. 1: Antibody titres in broilers having high levels of maternal antibody at day 0.

- 5 Fig. 2a: Nucleotide sequences A-segments
- Fig. 2b: Nucleotide sequences B-segments
- Fig. 3a: Amino acid sequences polyproteins
- Fig. 3b: Amino acid sequence VP1
- Fig. 3c: Amino acid sequence VP5

- 10 Fig. 4: Plasmid drawings
- Fig. 5a: Construction of pHB36-vvVP2
- Fig. 5b: Construction of pHB36-vvVP3
- Fig. 5c: Construction of pHB36-vvVP4
- Fig. 5d: Construction of pHB36-s2VP3
- 15 Fig. 5e: Construction of pHB36-s2VP3C
- Fig. 5f: Construction of pHB36-s2VP3N
- Fig. 5g: Construction of pHB60-s2VP3C1

- Fig. 6:** Comparison of the amino acid sequences of the VP3 encoding parts of the cDNAs of the cell culture adapted serotype I classical strain CEF94 and the wild-type serotype II strain TY89. The sequence of the CEF94 protein is presented, while only those amino acids which differ from the CEF94 sequence are given for TY89. Identical amino acids are represented by a dot. Plasmid pHB36-s2VP3 encodes the entire given amino acid sequence of serotype II (TY89). The nucleotide sequences of the A-segment cDNA's, which were used to deduce the amino acid sequences, have been deposited in the GenBank database: A-segment CEF94 (AF194428, full-length) and TY89 (xxxxx, partially). The position of the SacII and ScaI sites in the corresponding cDNA (see Fig. 7) is indicated.
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- 25
- 30

- Fig. 7:** Schematic representation of plasmids containing the full-length serotype I (CEF94) IBDV A-segment cDNA (pHB-36W), and mosaic full-length A-segment cDNAs (pHB36-s2VP3N, -s2VP3C and -s2VP3) based upon the genome of serotype I (CEF94) and serotype II (TY89) IBDV strains. Open boxes represent open reading frames based upon serotype I cDNA, while shaded boxes represent serotype II cDNA. Several genetic elements such as
- 35

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the remaining part of the polyprotein, the VP5 ORF, T7 RNA polymerase promoter and terminator sequences and the Hepatitis Delta Virus Ribozyme (HDVR) and are only shown for pHB-36W but these are also present at the same positions in the other
5 plasmids.

Fig. 8: SDS-PAGE analysis of IBDV proteins. a) Autoradiogram of a coupled in vitro transcription/translation reaction using plasmids containing either the full-length A-segment sequence of CEF94 (lane 1: pHB-36W) or mosaic full-length A-segment sequences (lane 2: pHB36-s2VP3, lane 3: pHB36-s2VP3N, or lane 4: pHB36-s2VP3C). The position of the viral (precursor) proteins are indicated on the right hand side, while the position of marker proteins (¹⁴C-labelled Amersham Rainbow marker) and their sizes (kDa) are indicated on the left hand side. b) Western blot analysis of purified IBDV of serotype I (lane 1: CEF94) and serotype II (lane 2: TY89). VP3 proteins (indicated by arrows) were visualized using a VP3 specific monoclonal antibody. The position of marker proteins (pre-stained Amersham Rainbow marker) and their sizes (kDa) are indicated on the right hand side. c) Autoradiogram of immunoprecipitated ³⁵S-methionine labeled IBDV proteins. In vitro synthesized viral proteins originating from pHB-36W (lane 1), pHB36-s2VP3 (lane 2), pHB36-s2VP3N (lane 3), or pHB36-s2VP3C (lane 4) were immunoprecipitated using either a polyclonal antibody directed against serotype I VP3 and VP4 (PaVP3/4), or monoclonal antibodies specific for VP3 of either serotype I (Mab IV^{SI}) or serotype II (Mab VII^{SII}).
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Fig. 9: Single step growth curves of rCEF94 and mCFE94-s2VP3C. QM5 cells were infected with IBDV (m.o.i. = 5, T = 0h) for one hour, washed four times, and covered with fresh medium. Samples were taken from the supernatant at different time points and the amount of IBDV (TCID₅₀ per ml) was determined.
30
35 Error bars represent standard deviations.

Fig. 10: Detection of VP3 antigen in (m)IBDV infected QM5 cells using different monoclonal antibodies. Fresh monolayers of QM5

cells were infected with either rCEF94, mCEF94-s2VP3, -s2VP3N, or -s2VP3C and incubated during 48h. Different monoclonal antibodies specific for VP3 of IBVD were used to assess the reactivity with the different mosaic viruses in an IPMA. An overview of the IPMA results of all used monoclonal antibodies is given in Table 11.

Fig. 11: Schematic representation of the plasmids containing the full-length (hybrid) A-segment cDNA sequences. The T7 promotor sequence, the hepatitis delta virus ribozyme (HDR), and the T7 terminator is only given for plasmids pHB36W and pHB60, but is present in each plasmid. Open boxes indicate CEF94 cDNA, shaded boxes indicate D6948 cDNA, while black boxes indicate TY89 cDNA.

Fig. 12: Western blot analysis of protein samples isolated 24h after transfection of FP-T7 infected QM-5 cells with either pHB-36W (1), pHB36-s2VP3 (2), pHB36-s2VP3N (3), pHB36-s2VP3C (4), pHB-60 (5), pHB60-s2VP3N (6), pHB60-s2VP3C1 (7), pHB60-s2VP3C3 (8). Four SDS-PAGE gels, containing equal amounts of cell extracts, were blotted onto nitrocellulose, and VP3 was detected using the four indicated Mabs. Size markers (Rainbow marker, Amersham) are indicated in kDa on the left.

Fig. 13: Comparison of the amino acid sequence of VP3 of the classical, cell culture adapted serotype I CEF94 strain, the very virulent serotype I D6948 strain and the wild-type serotype II TY89 strain. The sequence of the CEF94 protein is presented, while only those amino acids that differ from the CEF94 sequence are given for D6948 and TY89. The positions of the SacII and ScaI endonuclease restriction sites in the corresponding cDNA (see Fig. 11) are indicated.

Fig. 14: Formalin fixed bursa sections stained with Hematoxylin-Eosin (H & E). Layer-type SPF chickens infected with the indicated virus (upper right corner) at day 21 were euthanized at day 13 post infection, and bursas were examined.

The overall damage of the follicular structure was determined according to (Bayyari et al., 1996) and is given in Table 13.

Fig. 15: Resulting data of the competition Elisa's. The CEF94

5 VP3 antigen was coated, and a diluted serum sample (taken at 13 days post infection) of chickens infected with the indicated virus was mixed with either Mab IV^{SI} or I/G4^{SI}. The amount of bound Mab was determined using Rabbit-anti-Mouse conjugated with peroxidase and a TMB as substrate.

10

Fig. 16: Amino acid comparison between the different ORFs of

the cDNAs of wild-type vvIBDV isolate D6948 and the cell culture adapted classical isolate CEF94. The complete sequence of the D6948 proteins is given, while only those amino acids

15 which differ from the D6948 sequence are given for CEF94 (below the D6948 sequence). The nucleotide sequences of the A- and B-segments, which were used to deduce the amino acid sequences,

can be found in the GenBank database (accession numbers are given between parenthesis: A-segment D6948 (AF240686) and CEF94

20 (AF194428) and B-segment D6948 (AF240687), and CEF94

(AF194429). A) amino acid sequence encoded by the first ORF (VP5) of the A-segments, B) amino acid sequence encoded by the second ORF (polyprotein) of the A-segment. The VP4 of the polyprotein (underlined) is preceded by pVP2, while VP3 is

25 located at the C-terminus (see Fig. 17). The putative cleavage sites between pVP2 and VP4, and between VP4 and as suggested by (18) have been used. Only recently it was shown that the actual

cleavage sites are most likely located between amino acids 512-513 (pVP2-VP4) and 755-756 (VP4/VP3) (27). C) amino acid

30 sequence encoded by the single ORF encoded by the B-segment (VP1/VPg). Dashes are given in case corresponding amino acids are missing. Amino acid changes which are found in all vvIBDV sequences are given in bold face. Amino acid residues that are reported to be involved in adaptation to non B-lymphoid cells

35 are given in italics.

Fig. 17: Schematic representation of the plasmids containing the full-length cDNA sequence of the A-segment (pHB-60) and B-

segment (pHB-55) of the wild-type very virulent IBDV isolate D6948. The cDNA sequence is preceded by a T7 promotor sequence and is followed by the hepatitis delta virus ribozyme (HDR), and a T7 terminator. The different ORFs are represented by open boxes.

Fig. 18: Autoradiogram of an SDS-PAGE analysis of a coupled in vitro transcription/translation reaction. A) Full-length A-segment plasmids of attenuated classical IBDV isolate CEF94 (lane 1) and wild-type very virulent IBDV isolate D6948 (lane 2). B) Full-length B-segment plasmids of CEF94 (lane 1) and D6948 (lane 2). C) Full-length A-segment CEF94 (lane 1) and full-length A-segment plasmid of the classical attenuated IBDV isolated (pHB-36) in which either pVP2 (pHB36-vvVP2, lane 2), VP3 (pHB36-vvVP3, lane 3), or VP4 (pHB36-vvVP4, lane 4) has been exchanged. The positions of the viral proteins are indicated on the right. The sizes of the marker proteins (Rainbow marker, Amersham) are indicated on the left.

Fig. 19: Detection of IBDV using the VP3/4 polyclonal antiserum. Samples of supernatant of different plasmid transfections were used to infect QM5 or primary bursa cells. After infection the QM5 cells were incubated for 24h, while the primary bursa cells were incubated for 48h. IBDV proteins were visualized by performing an Immuno Peroxidase Monolayer Assay.

Fig. 20: Single step growth curves of CEF94, rCEF94 and srIBDV-CADB. QM5 cells were infected with IBDV (m.o.i. = 5, T = 0h) for one hour, washed three times, and covered with fresh medium. At different time points samples were taken from the supernatant and the amount of IBDV (TCID₅₀ per ml) was determined. The TCID₅₀ values at each time point is the mean of three independent experiments; error bars represent standard deviations.

Fig. 21: Schematic representation of the construction of a mosaic PCR fragment having a middle part originating from D6948

5 cDNA (shaded boxes), and flanking parts originating from CEF94
cDNA (open boxes). For the construction of pHB-36-vvVP2 we
first generated PCR fragments VP2a, VP2b, and VP2c. These PCR
fragments were purified and subsequently used as template in a
fusion PCR, yielding PCR fragment VP2d (17). This PCR fragment
was subsequently purified and used to replace the corresponding
part of the CEF94 A-segment cDNA, by using the indicated *EcoRI*
and *SacII* restriction sites. pHB36-vvVP3 and -vvVP4 were
10 generated using the same approach using different primers to
generate the PCR fragments and different restriction sites to
introduce the PCR fragments in the full-length A-segment clone
(see Material and Method section and Table 16).

Fig. 22: Detection of mosaic IBDV using VP3/4 polyclonal
15 antiserum. Samples of supernatant of different plasmid
transfections were used to infect QM5 and primary bursa cells.
After infection, the QM5 cells were incubated for 24h, while
the primary bursa cells were incubated for 48h (see Fig. 19).
In contrast to the negative control (mock-infected), in which
20 no B-lymphoid cells stained positive (data not shown), we
found several stained B-lymphoid cells scattered throughout
the culture tissue dish in the IPMA shown in the lower panel.

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Table 1 Classification of live IBDV vaccines used to induce active protection in young chickens which are passively protected by maternal IBDV antibodies.

5

Type of vaccine (live IBD virus)	Ability to induced an immune response when IBDV antibody titers are equal or below	Immunosuppressive
Mild	50-100	No
Intermediate	100-200 *	No
Strong	500 *	Yes

* The Animal Health Service (Deventer, The Netherlands) uses an Idexx Elisa value of 128 (2log7) as the maximum titer for the use of live intermediate vaccins and a value of 512 (2log9) for strong vaccines.

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Table 2 Primers (oligonucleotides) used for sequence determination, in RT-PCR or PCR reactions. Nucleotides which are unable to hybridize with wild-type IBDV genomes are given in small face. Primers which are specific

5 either for the serotype II (s2) or very virulent (vv) genome are indicated.

Name	Sequence	Position
Anchor	cacgaattcactatcgattctggatccttc	-
Anchor Primer	gaaggatccagaatcgatag	-
ANC0	GGGGACCCGCGAACGGATC	A: -1/-18
ANC1	GGGGACCCGCGAACGG	A: -1/-16
T7AC0	ggaattctaatacgactcactataGGATACGATCGGTCTGAC	TCCE/23
BNC1	GGGGCCCCCGCAGG	B: -1/-15
T7BC1	ggaattctaatacgactcactataGGATACGATGGGTCTGAC	BC1/21

Table 3 Nucleotide sequence corresponding to the 5'- and 3'-termini of the coding strands of the two genomic segments of IBDV (CEF94). Numbers behind specific sequences indicate the number of times each sequence was obtained.

5'-terminus of the A-segment coding strand	Complementary sequence of the 5'-terminus of the B-segment coding strand	Complementary sequence of the 5'-terminus of the B-segment non-coding strand
5'UGAUACGAUC>>>	>>>CGGG ³	5'UGAUACGAUC>>> (2x)
5'AGAUACGAUC>>>	>>>CGGGUCCC ³	5'GGAUACGAUC>>> (5x)
5'GGAUACGAUC>>> (7x)	>>>CGGGUCCCC ³	>>>CGGGGCCCC ³ (2x)
	>>>CGGGUCCCC ³ (6x)	>>>CGGGGCCCC ³ (2x)
	>>>CGGGUCCCCC ³	>>>GGUGGCCCCC ³
	>>>CGGGUCCCCCU ³	>>>CGGGGCCCCCCC ³
	>>>CGGGUCCCCCCC ³	>>>CGGGGCCCCCCC ³
Consensus 5'GGAUACGAUC>>>	>>>CGGGUCCCC ³ (nt 3260)	5'GGAUACGAUC>>> (nt 2827)

Table 4 Description of the used plasmids

Name	Based on	Description
pUC18-Ribo	pUC18	Contains the <i>SmaI-XbaI</i> fragment of pTV-2A
pHB-36A	pUC18-Ribo	Contains the consensus cDNA sequence of the A-segment of CEF94 (see Fig. 2a)
pHB-36W	pHB-36A	An artificially introduced <i>KpnI</i> -site (genetic tag) in the 3'-UTR of the CEF94 A-segment encoding cDNA (Fig. 2)
pHB-36	pHB-36A	Contains a lethal amino acid substitution in the VP4 part of the polyprotein (V582A)
pHB-60	pUC18-Ribo	Contains the consensus cDNA sequence of the D6948 A-segment (see Fig. 2a)
pHB-34Z	pUC18-Ribo	Contains the consensus cDNA sequence of the CEF94 B-segment (see Fig. 2b)
pHB-55	pUC18-Ribo	Contains the consensus cDNA sequence of the D6948 B-segment (see Fig. 2b)
pSV-TY89-VP3	pGEM-Teasy	Contains the consensus cDNA of TY89 encoding the entire VP3 (A-segment, see Fig. 2)
pHB36-vvVP2	pHB-36W	Contains D6948 A-segment cDNA which encodes the entire VP2 (453 amino acids)
pHB36-vvVP3	pHB-36W	Contains D6948 A-segment cDNA which encodes the entire VP3 (289 amino acids)
pHB36-vvVP4	pHB-36W	Contains D6948 A-segment cDNA which encodes the entire VP4 (270 amino acids)
pHB36-s2VP3	pHB-36W	Contains TY89 A-segment cDNA which encodes the entire VP3 (289 amino acids)
pHB36-	pHB-36W	Contains TY89 A-segment cDNA which encodes the C-terminal part (122 amino

s2VP3C		acids) of VP3
pHB36-	pHB-36W	Contains TY89 A-segment cDNA which encodes the N-terminal part (168 amino
s2VP3N		acids) of VP3
pHB60-	pHB-60	Contains cDNA encoding a mosaic polypeptide (D6948 (1-543 AA), CEF94 (544-889
s2VP3C1		AA), and TY89 (890-1012 AA). The 5'-UTR is derived from D6948, while the 3'-UTR is
		derived from CEF94. An unique <i>Kpn</i> I-site (genetic tag) is furthermore present in the
		3'-UTR

Table 5 Description of the generated rIBDV, srIBDV, and mIBDV. The ability of these viruses to infect QM5 or primary bursa cells was examined in an immuno peroxidase monolayer assay (IPMA) using either polyclonal serum directed against VP3 or monoclonal antibodies directed against VP2 of IBDV serotype I (1.4), VP3 of serotype II (T-75), or VP3 of serotype I (B10 and C3); nd means not determined.

IBDV virus	Derived from plasmids		Replication on					
	A-segment	B-segment	QM5 cells	Bursa	1.4	T75	B10	C3
rCEF94	pHB-36W	pHB-34Z	+	+	+	-	+	+
rD6948	pHB-60	pHB-55	-	+	+	-	+	+
srIBDV-DACB	pHB-60	pHB-34Z	-	+	nd	nd	nd	nd
srIBDV-CADB	pHB-36W	pHB-55	+	nd	nd	nd	nd	nd
mCEF94-vvVP2	pHB36-vvVP2	pHB-34Z	-	+	nd	nd	nd	nd
mCEF94-vvVP3	pHB36-vvVP3	pHB-34Z	+	nd	nd	nd	nd	nd
mCEF94-vvVP4	pHB36-vvVP4	pHB-34Z	+	nd	nd	nd	nd	nd
mCEF94-s2VP3	pHB36-s2VP3	pHB-34Z	+	nd	+	+	-	+/-
mCEF94-s2VP3C	pHB36-s2VP3C	pHB-34Z	+	nd	+	+	-	+/-
mCEF94-s2VP3N	pHB36-s2VP3N	pHB-34Z	+	nd	+	+/-	+	-
mD6948-s2VP3C1	pHB60-s2VP3C1	pHB-55	-	+	+	+	-	+/-

Table 6 Clinical data of 21-day old chickens infected with wild-type, rIBDV, srIBDV or mIBDV isolates (12 groups of 10 chickens). Each chicken was inoculated with 1000 ELD₅₀ IBDV, except for the negative control group (PBS), and each group was kept in a separate isolator. The bursa and body weight of euthanized chickens was determined at nine days post infection. Standard deviation is given between brackets, together with the number of animals (n) used for determination of the given numbers. The bursa/body weight ratio for each animal was calculated and mean values (standard deviation) per group are given.

IBDV virus	Number of deads (after 9 days)	Body weight (grams)	Bursa weight (grams)	Bursa/Body weight (*1000)
PBS	0	305 (29, n=10)	1.9 (0.4, n=10)	6.1 (1.2)
CEF94	0	341 (16, n=6)	2.0 (0.6, n=6)	6.0 (1.8)
D6948	3	245 (56, n=7)	0.4 (0.1, n=7)	1.7 (0.6)
rCEF94	0	317 (15, n=6)	1.3 (0.5, n=6)	4.2 (1.3)
rD6948	5	261 (24, n=5)	0.4 (0.1, n=5)	1.7 (0.2)
srIBDV-DACB	2	263 (35, n=8)	0.4 (0.1, n=8)	1.5 (0.3)
srIBDV-CADB	0	314 (13, n=6)	1.8 (0.8, n=6)	5.7 (2.7)
mCEF94-vvVP2	0	309 (27, n=6)	0.6 (0.2, n=6)	1.9 (0.4)
mCEF94-vvVP3	0	325 (33, n=6)	2.0 (0.3, n=6)	6.2 (0.7)
mCEF94-vvVP4	0	330 (23, n=6)	1.5 (0.5, n=6)	4.4 (1.3)
mCEF94-s2VP3C	0	320 (11, n=6)	1.3 (0.4, n=6)	4.1 (1.3)
mD6948-s2VP3C1	0	315 (26, n=6)	0.6 (0.2, n=6)	1.9 (0.6)

Table 7: Origin and phenotype of the IBDV isolates

Isolate	Reference	Virulence
D6948	Boot et al., unpublished	Very virulent
rD6948	Boot et al., unpublished	Very virulent
UK661	Brown and Skinner, 1996	Very virulent
5123	Ter Huurne et al., unpublished	Very virulent
96-B4	Ter Huurne et al., unpublished	Avirulent
96-C4	Ter Huurne et al., unpublished	Avirulent
96-C5	Ter Huurne et al., unpublished	Avirulent
96-C6	Ter Huurne et al., unpublished	Very virulent
97-B3	Ter Huurne et al., unpublished	Avirulent
97-B4	Ter Huurne et al., unpublished	Very virulent
97-B5	Ter Huurne et al., unpublished	Very virulent
97-B6	Ter Huurne et al., unpublished	Very virulent
Zoontjes	Ter Huurne et al., unpublished	Very virulent
Hungary	Ter Huurne et al., unpublished	Very virulent
OKYM	Yamaguchi et al., 1996	Very virulent
OKYMT	Yamaguchi et al., 1996	Avirulent
TKSM	Yamaguchi et al., 1996	Very virulent

TKSMT	Yamaguchi et al., 1996	Avirulent
HK46	Lim et al., 1999	Very virulent
HK46-	Lim et al., 1999	Not determined
NT		

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Table 8:

Amino acid sequence of the hypervariable region of VP2 of different IBDV isolates. The sequence of the hydrophilic regions (underlined) and the hydrophobic region of very virulent isolate D6948 (amino acid 214 to 328) is used as parental isolate for alignment of the other sequences. Identical amino acid are represented by a dash.

D6948	214AADDYQFSSQYQAGGVTTILFSANIDAITSLSIGGELVFQTSVQGLILGATIIYLIGFDGTAVITRAVAADNGLTACTDNLMPFNIVIPTSBITQPIITSIKLEIVTSKSGGQAGDOMSW328
rD6948	-----
UK661	-----
5123	-----
96-B4	-----V-----V-----T-----N-----T-----L-----N-----
96-C4	-----P-----L-----N-----A-----N-----T-----S-----T-----I-----N-----
96-C5	-----P-----V-----V-----V-----Y-----T-----L-----L-----N-----
96-C6	-----
97-B3	-----
97-B4	-----
97-B5	-----
97-B6	-----
Zoontjes	-----T-----V-----
Hungary	-----
OKYM	-----
OKYMT	-----T-----N-----T-----F-----
TKSM	-----
TKSMT	-----H-----D-----T-----N-----T-----
HK46	-----
HK46-NT	-----N-----T-----



Table 9

Virus	A-segment		B-segment		Feature
	plasmid		plasmid		
rCEF94	pHB-36W		pHB-52		Rescued CEF94
mCEF94-s2VP3	pHB36-s2VP3		pHB-52		Rescued mosaic IBDV, which encodes a polypeptide consisting of amino acid 1-723 originating from CEF94 and amino acids 724-1012 originating from TY89; 760 nucleotides have been exchanged of which 102 are different (13%).
mCEF94-s2VP3N	pHB36-s2VP3N		pHB-52		Rescued mosaic IBDV, which encodes a polypeptide consisting of amino acid 1-723 and 857-1012 originating from CEF94 and amino acids 724-856 originating from TY89; 398 nucleotides have been exchanged of which 65 are different (16%).
mCEF94-s2VP3C	pHB36-s2VP3C		pHB-52		Rescued mosaic IBDV, which encodes a polypeptide consisting of amino acid 1-856 originating from CEF94 and amino acids 857-1012 originating from TY89; 371 nucleotides have been exchanged, of which 37 are different (10%)

Table 10

Virus	TCID ₅₀ after transfection			Maximum* TCID ₅₀
	Exp. 1	Exp. 2	Exp. 3 Average	
rCEF94	4.9	5.6	4.7	5.1
mCEF94-s2VP3	2.0	0.3	1.0	1.1
mCEF94-s2VP3N	3.0	3.5	2.9	3.1
mCEF94-s2VP3C	5.0	5.0	4.3	4.8

* Determined after 3 passages of initially rescued virus

Table 11

Monoclonal antibody	Directed against	Reaction of IBDV proteins in an immuno peroxidase monolayer assay				
		CEF94	TY89	rCEF94	mCEF94-s2VP3	mCEF94-s2VP3N
1.4	VP2, serotype I	+	-	+	+	+
9.8	VP2, serotype I	+	-	+	+	+
9.7	VP3, serotype I	+	+	+	+	+
IV	VP3, serotype I	+	-	+	-	-
I/G4	VP3, serotype I	+	-	+	-	-
VII	VP3, serotype II	-	+	-	+	+
17/80	VP3, serotype I	+	+	+	+	+

Table 12 Description of the used viruses

Name	Based on plasmid ²	Main features
CEF94	n.r.	Serotype I, classical, cell culture adapted IBDV strain
D6948	n.r.	Serotype I, very virulent IBDV field isolate
TY89	n.r.	Prototype serotype II IBDV field isolate
rCEF94	pHB-36W + pHB-34Z	Rescued CEF94: classical, cell culture adapted IBDV
rD6948	pHB-60 + pHB-55	Rescued D6948: very virulent IBDV
mCEF94-s2VP3	pHB36-s2VP3 + pHB-34Z	Rescued mosaic IBDV having a hybrid polyprotein; amino acid 1-723 of CEF94 and 723- 1012 of TY89
mCEF94- s2VP3N	pHB36-s2VP3N + pHB-34Z	Rescued mosaic IBDV having a hybrid polyprotein; amino acid 1-723 and 857-1012 of CEF94 and 723-856 of TY89
mCEF94- s2VP3C	pHB36-s2VP3C + pHB-34Z	Rescued mosaic IBDV having a hybrid polyprotein; amino acid 1-856 of CEF94 and 857- 1012 of TY89
mD6948-s2VP3N ¹	pHB60-s2VPN + pHB-55	mosaic IBDV cDNA encoding a hybrid polyprotein; amino acid 1-723 and 857-1012 of D6948 and 723-856 of TY89
mD6948-s2VP3C1	pHB60-s2VP3C1 + pHB-55	Rescued mosaic IBDV having a hybrid polyprotein; amino acid 1-543 of D6948, 544-856 of CEF94, and 857- 1012 of TY89
mD6948- s2VP3C3	pHB60-s2VP3C3 + pHB-55	Rescued mosaic IBDV having a hybrid polyprotein; amino acid 1-856 of D6948 and 857- 1012 of TY89)

1: this virus could not be rescued

2: n.r. not relevant

Table 13: Experimental data of the chicken infection experiments 1 and 2

Virus	CODE	Mortality ¹	Index Antibody titers ²	VN titers ³	Bursa weight (gr) ⁴	Bursa/Body weight ⁵	HBLs ⁶
Exp. 1	99.16						
PBS	Gr. 1	0/10	0 (0, 10, 0)	0.0 (0.0)	1.9 (0.4, 10)	6.1 (1.2)	1.0 (0.0, 9)
CEF94	Gr. 2	0/10	207 (507, 6, 1)	6.5 (1.2)	2.0 (0.6, 6)	6.0 (1.8)	1.6 (1.1, 10)
rCEF94	Gr. 4	0/10	260 (290, 6, 3)	9.7 (1.8)	1.3 (0.5, 6)	4.2 (1.3)	1.9 (0.7, 10)
mCEF94-s2VP3C	Gr. 11	0/10	824 (433, 6, 6)	10.8 (1.5)	1.3 (0.4, 6)	4.1 (1.3)	1.1 (0.3, 10)
D6948	Gr. 3	3/10	1626 (690, 7, 7)	10.3 (1.4)	0.4 (0.1, 7)	1.7 (0.6)	5.0 (0.0, 7)
rD6948	Gr. 5	5/10	1549 (791, 4, 4)	10.3 (1.5)	0.4 (0.1, 5)	1.7 (0.2)	5.0 (0.0, 4)
mD6948-s2VP3C1	Gr. 12	0/10	956 (326, 6, 6)	10.8 (1.2)	0.6 (0.2, 6)	1.9 (0.6)	5.0 (0.0, 8)
Exp. 2	99.18						
PBS	Gr. 1	0/15	0 (0, 15, 0)	0.0 (0.0)	2.3 (0.7, 15)	6.1 (1.8)	1.4 (1.3, 10)
D6948	Gr. 2	8/15	2489 (948, 7, 7)	11.7 (1.6)	0.3 (0.2, 7)	0.8 (0.7)	5.0 (0.0, 8)
rD6948	Gr. 3+6	7/30	2308 (905, 23, 23)	10.0 (1.5)	0.3 (0.1, 23)	1.0 (0.4)	5.0 (0.0, 20)
mD6948-s2VP3C1	Gr. 4	1/15	2122 (1300, 14, 14)	9.0 (1.8)	0.9 (0.2, 14)	2.7 (0.7)	4.7 (0.7, 10)
mD6948-s2VP3C3	Gr. 5	0/15	1760 (880, 15, 15)	8.8 (1.6)	0.5 (0.1, 15)	1.6 (0.4)	5.0 (0.0, 10)

1 Accumulated number of mortalities during the first 5 days p.i./total number of challenged birds

2 Average value (standard deviation, number of chicken samples used, number of positive samples)

3 The presence of neutralizing antibodies was determined using the CEF94 IBDV strain; titers are given as 2Log values (standard deviation)

4 Average bursa weight (*1000) of chickens in each group is given (standard deviation, number of animals)

5 The bursa weight / body weight ratio for each animal was calculated and the average value (standard deviation) per group is given

6 Average histopathologic bursal lesion score (HBLs) according to Ravvari *et al* (1994) * who survived the infection is given (standard deviation, number of bursae scored)

Table 14 IBDV specific antibody titers (Idexx) before and after infection with mD6948-s2VP3C3

Days p.h. ¹	Group 1 ²	Group 2	Group 3	Group 4	Group 5
7	1997 (300, 17, 17)	2991 (543, 17, 17)	3332 (691, 18, 18)	3657 (676, 17, 17)	3970 (446, 14, 14)
15	18 (75, 17, 1)	361 (101, 17, 16)	576 (65, 18, 18)	899 (150, 17, 17)	1283 (154, 14, 14)
18	infection	infection	infection	infection	infection
22	0 (0, 17, 0)	0 (0, 17, 0)	0 (0, 17, 0)	0 (0, 17, 0)	95 (261, 14, 2)
25	391 (600, 12, 4)	822 (1261, 12, 5)	477 (1166, 13, 2)	479 (750, 12, 4)	516 (1084, 9, 3)
32	1587 (852, 12, 11)	1468 (746, 12, 12)	367 (882, 12, 2)	0 (0, 11, 0)	0 (0, 9, 0)
42	3241 (905, 11, 11)	2877 (1177, 10, 10)	149 (227, 11, 4)	132 (234, 11, 2)	2809 (633, 9, 9)

1 p.h. = post hatch

2 The given antibody titers are the average of the positive animals with the standard deviation (first value between brackets), number of animal samples used (second value between brackets) and number of samples positive (third value between brackets) of values determined in duplo for each chicken in the group at a given day post hatch (p.h.)

Table 15 IBDV neutralizing antibody titers determined before and after infection with mD6948-s2VP3C3

Days p.h. ¹	Group 1 ²	Group 2	Group 3	Group 4	Group 5
15	7.2 (0.8, 17)	8.8 (1.0, 17)	9.3 (1.4, 15)	9.9 (1.4, 17)	9.9 (1.2, 14)
18	Infection	Infection	Infection	Infection	Infection
25	5.8 (1.9, 12)	4.8 (1.6, 12)	6.4 (1.5, 8)	7.6 (1.6, 7)	7.3 (0.9, 9)
32	10.9 (1.1, 12)	10.8 (1.4, 12)	5.3 (1.2, 12)	5.4 (0.7, 11)	5.6 (0.7, 9)
42	9.8 (1.6, 11)	10.0 (1.3, 10)	3.6 (0.9, 11)	3.7 (0.7, 11)	9.3 (1.3, 9)

1 p.h. = post hatch

2 The given values are the average of the individual ²Log virus neutralizing antibody titers of animals belonging to the same groups. Between brackets is the standard deviation and number of sera used, respectively.

Table 16 Description of plasmids and viruses

Name	Based on plasmid*	Main feature
Plasmids		
pUC18-Ribo	pUC18	Contains the hepatitis delta virus ribozym
pHB-22R	pGEM-T	A-segment cDNA of D6948 (consensus sequence)
pHB-60	pUC18-Ribo	A-segment cDNA of D6948, including a genetic tag
pHB-36W	pUC18-Ribo	A-segment cDNA of CEF94, including a genetic tag
pHB-34Z	pUC18-Ribo	B-segment cDNA of CEF94 (consensus sequence)
pHB-55	pUC18-Ribo	B-segment cDNA of D6948 (consensus sequence)
pHB36-vvVP2	pHB-36W	pVP2 of CEF94 cDNA is replaced by pVP2 of D6948 (453 amino acids)
pHB36-vvVP3	pHB-36W	VP3 of CEF94 cDNA is replaced by VP3 of D6948 cDNA (289 amino acids)
pHB36-vvVP4	pHB-36W	VP4 of CEF94 cDNA is replaced by VP4 of D6948 cDNA (270 amino acids)
Viruses		
CEF94	n.r.	Classical, cell culture adapted IBDV isolate (derivative of PV1)
D6948	n.r.	Very virulent IBDV field isolate (The Netherlands, 1989)
rCEF94	pHB-36W + pHB-34Z	Rescued IBDV: classical, cell culture adapted IBDV
rD6948	pHB-60 + pHB-55	Rescued IBDV: very virulent IBDV
srIBDV-CADB	pHB-36W + pHB-55	Rescued segment reassorted IBDV: (A-segment = CEF94, B-segment = D6948)
srIBDV-DACB	pHB-60 + pHB-34Z	Rescued segment reassorted IBDV: (A-segment = D6948, B-segment = CEF94)
mCEF94-vvVP2	pHB36-vvVP2 + pHB-34Z	Rescued mosaic IBDV, pVP2 originates from D6948, the remaining parts from CEF94
mCEF94-vvVP3	pHB36-vvVP3 + pHB-34Z	Rescued mosaic IBDV, VP3 originates from D6948, the remaining parts from CEF94
mCEF94-vvVP4	pHB36-vvVP4 + pHB-34Z	Rescued mosaic IBDV, VP4 originates from D6948, the remaining parts from CEF94

* n.r.: not relevant

Table 17: Experimental data of 21-day-old chickens challenged with 1000 ELD₅₀ of wild-type, rIBDV, srIBDV, or mIBDV isolates.

Virus*	Mortality ¹	Bursa weight (gr) ²	Bursa/body weight ³ (*1000)	HBL.S ⁴	IBDV antigens ⁵	Antibody titers ⁶ (Idexx)
PBS	0/10	1.9 (0.4, 10)	6.1 (1.2)	1.0 (0.0, 9)	-	0 (0, 10, 0)
CEF94	0/10	2.0 (0.6, 6)	6.0 (1.8)	1.6 (1.1, 10)	+	207 (507, 6, 1)
D6948	3/10	0.4 (0.1, 7)	1.7 (0.6)	5.0 (0.0, 7)	nd	1626 (690, 7, 7)
rCEF94	0/10	1.3 (0.5, 6)	4.2 (1.3)	1.9 (0.7, 10)	+	260 (290, 6, 3)
rD6948	5/10	0.4 (0.1, 5)	1.7 (0.2)	5.0 (0.0, 4)	+	1549 (791, 4, 4)
srIBDV-DACB	2/10	0.4 (0.1, 8)	1.5 (0.3)	5.0 (0.0, 6)	+	833 (415, 8, 8)
srIBDV-CADB†	0/10	1.5 (0.4, 5)	4.7 (1.0)	1.9 (1.3, 10)	+	400 (619, 6, 2)
mCEF94-vvVP2	0/10	0.6 (0.2, 6)	1.9 (0.4)	5.0 (0.0, 10)	+	953 (186, 6, 6)
mCEF94-vvVP3	0/10	2.0 (0.3, 6)	6.2 (0.7)	1.4 (0.5, 10)	nd	454 (399, 6, 4)
mCEF94-vvVP4	0/10	1.5 (0.5, 6)	4.4 (1.3)	1.5 (0.5, 10)	+	431 (356, 6, 4)

1 Accumulated number of mortalities during the first 9 days p.i./total number of challenged birds

2 Average bursa weight (9 days p.i.) of chickens is given (standard deviation, number of animals)

3 The bursa/body weight ratio for each animal was calculated and the mean value (standard deviation) per group is given

4 Average histopathologic bursal lesion score (HBL.S) of birds who survived the challenge is given (standard deviation, number of bursae scored)

5 Presence of VP4/VP3 antigens in bursa coupes was determined (nd = not done)

6 IBDV Antibody titers (Idexx) of the chickens euthanised at day 9 p.i. (standard deviation, number of chickens used, number positive chickens).

* Viruses given in bold-face are unable to grow on QM5 cells

† The values of one chick of this group have been left out, as this chick had an unusually large bursa (3.4 gr)

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